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TITLE: Breast Cancer in African American Women: Molecular Analysis of Differences in Incidence and Outcomes

PRINCIPAL INVESTIGATOR: Agnes A. Day, Ph.D.
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ABSTRACT

The DOD funded HBCU/MI Partnership Training award entitled "Breast Cancer in African American Women: Molecular Analysis of Differences in Incidence and Outcomes" continues to provide quality research experiences to faculty and students and has developed into a highly rewarding collaboration that has expanded from the original 2 faculty at HUCC to include 6 experienced faculty and multiple students. At WRAIR, the original 2 has increased to include 3 new researchers and numerous support staff and students. We have had a total of 9 animal handling workshops attended by students and faculty interested in cancer research, several workshops on establishment of the various stages (virgin, pregnant and lactating) of normal breast organ cultures, workshop on imaging of live animals using luminescent-tagged cells, as well as small group collaborations/meetings on genomics, bioinformatics and proteomics. As a result of participating in our various workshops sponsored by this training grant, especially the in vivo imaging of live animals, Dr. Paul Wang was successful in receiving an HBCU-MI partnership grant from CDMRP-BCRP aimed at bringing this technology to Howard University. These workshops have been very popular with faculty and students performing both cancer and other basic science research. Additionally, our previous reports have detailed the presentation of four workshops on Grantsmanship, Grant Writing and Writing Successful Animal Protocols. We have amassed a multidisciplinary core of students, post-doctoral fellows and faculty who are poised to perform several collaborative studies on breast, and other cancers, as a result of training received from this program. We have jointly produced manuscripts yearly, made key presentations at conferences, published numerous abstracts and continue to expand the original specific aims of the proposal

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Progress Report

INTRODUCTION

The DOD funded HBCU/MI Partnership Training award entitled "Breast Cancer in African American Women: Molecular Analysis of Differences in Incidence and Outcomes" continues to provide quality research experiences to faculty and students and has developed into a highly rewarding collaboration that has expanded from the original 2 faculty at HUCC to include 6 experienced faculty and multiple students. At WRAIR, the original 2 has increased to include 3 new researchers and numerous support staff and students. We have had a total of 9 animal handling workshops attended by students and faculty interested in cancer research, several workshops on establishment of the various stages (virgin, pregnant and lactating) of normal breast organ cultures, workshop on imaging of live animals using luminescent-tagged cells, as well as small group collaborations/meetings on genomics, bioinformatics and proteomics. As a result of participating in our various workshops sponsored by this training grant, especially the in vivo imaging of live animals, Dr. Paul Wang was successful in receiving an HBCU-MI partnership grant from CDMRP-BCRP aimed at bringing this technology to Howard University. These workshops have been very popular with faculty and students performing both cancer and other basic science research. Additionally, our previous reports have detailed the presentation of four workshops on Grantsmanship, Grant Writing and Writing Successful Animal Protocols. We have amassed a multidisciplinary core of students, post-doctoral fellows and faculty who are poised to perform several collaborative studies on breast, and other cancers, as a result of training received from this program. We have jointly produced manuscripts yearly, made key presentations at conferences, published numerous abstracts and continue to expand the original specific aims of the proposal.

Our initial goals for the 4th year were as follows:

- To adapt the in vivo imager to be able to detect both luminescence and fluorescence
- To utilize the technology developed by Dr. Stubbs & Das (this grant, 2004) for creation of stable cell lines of African-American breast cancer cells constitutively expressing the GFP tag.
- To create and utilize a training pipeline of students who work jointly in our labs and develop skills to for their dissertation work.
- To continue to attract pre-doctoral students, post-doctoral fellows, medical students and faculty to breast cancer research via introductory symposia, hands-on workshops, specialized training and research opportunities.
- To perform both collaborative and individual breast cancer research at both sites, Howard University Cancer Center (HUCC) and Walter Reed Army Institute for Research (WRAIR), which would add to the knowledge base and ultimately lead to the understanding, cure and/or prevention of this disease.

Administrative successes:

- A full-time Junior faculty who was hired to work on BRCA 1 & 2 in clinical samples from African-American women, has now been promoted from Instructor to Assistant Professor, tenure-track in the Department of Microbiology at Howard University.
- We are in the process of securing a joint appointment as adjunct assistant professor appointment for Dr. Rasha Hammamieh (WRAIR) in the Department of Microbiology/HUCC.
- Monthly meetings were continued between the key investigators and coordinator at HUCC and WRAIR. Meeting sites were alternated. These meetings were supplemented with e-mail and Tele-conferencing.

KEY ACCOMPLISHMENTS

Honors, Awards and Achievements

Faculty

Agnes A. Day:

a) Appointed Chairwoman, Dept of Microbiology, Howard Univ Coll. Medicine (7-05)
b) Reviewer, American Association for the Advancement of Science Policy Fellows Program. Washington, DC. March 1and 17, 2005
c) Reviewer, Dept of Defense, Prostate Cancer Research Prog, Tysons Corner, VA. April 05.
d) Reviewer, Dept of Defense, Breast Cancer Research Prog, Tysons Corner, VA, Sept. 2005.

John Stubbs

a) Travel Award, Gordon Research Conference on Small Integrin Binding Proteins, Big Sky, Montana, September 11-16, 2005.

Graduate/Medical Students

Jacquline A. Mason

4th Year Ph. D. Student, Microbiology

- Minority Scholar in Cancer Research Travel Award 2005 to attend and present at the 96th Annual Meeting, Anaheim, California
- Biotechnology 2005 Minority and Indigenous Fellows Award to attend the Bio 2005 Conference in Philadelphia, PA.
- Minority Scholar in Cancer Research Travel Award 2005 to attend the Breast Cancer Genetics Special Conference in La Jolla, California
- Outstanding Scholarly Publication Award 2005, given by Howard University Graduate School of Arts and Sciences, April, 2005.

Tamara Tatum-Broughton

6th Year Ph.D. Student, Microbiology

- Nomination for and selection as a facilitator of the Minority Affairs Booth at the 44th Annual Meeting for the American Society of Cell Biology (ASCB) in Washington, DC in December 2004.
- Selection as a 2005 American Association for Cancer Research (AACR) Minority Scholar. This award provided paid travel to and registration for the 96th Annual AACR Meeting in Anaheim, CA in April 2004.
- Participation as a member of the Selection Committee for the 2005 Biotechnology Institute/AMGEN Minority and Indigenous Fellows Program.
- Participation as a member of the Minority Health Disparities Panel Committee.
- Participation as a member of the Outreach and Education Committee for Biotechnology Industry Organization (BIO).
- Participation in the 2005 Biotechnology Institute/AMGEN Minority and Indigenous Fellows Program as the coordinator and moderator of evaluation process for the fellows and their mentors.

Charles E. Thompson, III

Howard University College of Medicine- Medical Student Year III

- Charles Thompson, Jacquline Mason, Haile Yancy and Agnes Day. 2005. Comparative Study of Metastatic Progression and Gene Expression between African American and Caucasian Women with Breast Cancer. Howard University College of Medicine's Medical Staff and Student's Research Day. October 9, 2005

Grants

a) NCI –U54 (L. Adams-Campbell, PI) **Agnes A. Day**, Pilot Project, Investigator, HUCC
Neal Fedarko, Co-Investigator, Johns Hopkins
"Molecular Analysis of Co-Expression of Matrix Metalloproteinases and SIBLINGS in African American-vs-Caucasian Women."
Howard /Hopkins Partnership, Pilot Project $150,000. 9/1/04-8/31/06

b) SO6 GM 08016-32 NIGMS SCORE
(Program Director): George Littleton, Ph.D.
(Investigator): **John T. Stubbs III, Ph.D.**
Source: Department of Health and Human Services, NIH
Title of Subproject: Effects of extracellular matrix proteins on prostate cancer cells
Dates of Approved Project: 9/1/02-7/31/06

c) Submitted. 2005 Co-Investigator **John T. Stubbs III**

Molecular MR Imaging of Breast Cancer for Early Diagnosis and Immunotherapy Evaluation. A Multidisciplinary Training Program in Molecular Biology and MR Imaging. Lian Shan, MD, Ph.D, Principal Investigator; Source: Army

- Trained HU students and young faculty in specific technologies which led directly to grant applications, publications and new research projects.
- Presented research accomplishments in national and international meetings
- Published 2 full manuscripts in peer reviewed journals, 2 full manuscripts are in preparation for peer review, published numerous abstracts in national and international meetings.
- Personnel involved obtained prestigious travel grant awards to national and international meetings;
- PI was promoted to Permanent Chairperson of Microbiology (July 2005).
- Continued with 2 new cancer-related courses in the Howard University Cancer Center
- Publication of a highly successful experiment for testing anticancer drugs in breast cancer cells (designed for high school level students from DC inner city public schools) Sept 2005.
- Submitted 2 new grant proposal applications
- Continued collaborative research projects between HUCC and WRAIR.
- Made major progress towards pinpointing differences between breast cancer cells from African-Americans and Caucasians.

REPORTABLE OUTCOMES

Published Journal Articles

a) Tesema, Y., Raghavan, D., and Stubbs III, J. (2005) Bone cell viability on methacrylic grafted and collagen immobilized porous poly (3-hydroxybuterate-co-3-hydroxyvalerate) J. Appl. Polymer Sci. 98:1916-1921
http://www3.interscience.wiley.com/cgi-bin/fulltext/112092854/PDFSTART
NOTE: This article sets up an artificial bone model and provides a potential metastasis model for specific breast cancer cell lines established from African American women.

b) R. Hammamieh, M. Anderson, K. Carr, C. Tran, D. Yourick and Marti Jett (2005). Students Investigating the Antiproliferative effects of synthesized drugs on mouse mammary tumor cells. Cell Biology Education 4:221-234.

c) R. Hammamieh, N. Chakraborty, M. Barmada, R. Das and Marti Jett (2005). Expression patterns of fatty acid binding proteins in breast cancer cells. J. Experimental Therapeutics and Oncology 5:133-143.

Manuscripts Submitted

L.Y. Berkeley, O.O. Kassim, A. Day, R. Kittles, W. Austin, L. Frederick, M. Coomes, J. Stubbs (2005) Phenotypic, Biochemical and Genetic Characterization of *Bacillus sp. BFAS* An Antifungal Producing Bacterium (Submitted to J. Canadian Microbiology). This extract may have anticancer activities for breast cancer.

Manuscripts in Preparation

a) Haile F Yancy[1,2], Sharla Peters[2], Charles E Thompson III[2], Jacquline A Mason[2], George K Littleton and[3] Agnes A Day*[2] Comparative Study of Gene Expression during Metastatic Progression between African American and Caucasian Women. In preparation for Cancer Research
b) Rasha Hammamieh, N. Chakraborty, S. Miller, Agnes Day, Marti Jett. Global genomic analysis of the effect of omega-3 and omega-6 fatty acids on breast cancer cells. Prepared for submission to J. Experimental Therapeutics and Oncology

Presentations

a) H. Liu, Y. T. Tesema, J. Stubbs III, D. Raghavan (2005) In vitro degradation of porous PHBV film. Number 611. Polymers for Bioactive Surfaces. Co-sponsored by The Division of Polymer Chemistry (POLY) and The Division of Biological Chemistry (BIOL). National American Chemical Society Meeting. Washington, DC http://www.polyacs.org/arcmeetings/washington.805.shtml

b) Mason, J.A., H. Yancy, A. Day. 2005. Differential expression of RECK and MMPs between African American and Caucasian breast cancer cell lines. Proceedings of the American Association for Cancer Research. Volume 46; April 2005. Philadelphia, Pennsylvania.
c) Charles Thompson, Jacquline Mason, Haile Yancy and Agnes Day. 2005. Comparative Study of Metastatic Progression and Gene Expression between African American and Caucasian Women with Breast Cancer. Howard University College of Medicine's Medical Staff and Student's Research Day. October 9, 2005
d) J. Stubbs III and B. Kifle (2005) Bone sialoprotein interactions with prostate cancer cells. The Gordon Research Conference on Small Integrin Binding Proteins, Big Sky, Montana.
e) Agnes A. Day, "SuperBugs": Emerging and Re-Emerging Infectious Diseases. Howard University's Mini-Medical School. Howard University, Washington, DC. October, 2004

f) Agnes A. Day, 2005." My Road to a Career in Science", Bowie State University, Bowie, MD, April 29, 2005
g) Agnes A. Day, 2005. "Microbiology and You". Public Science Day, American Association for the Advancement of Science. University of the District of Columbia, Washington, DC Feb. 17, 2005
h) Rasha Hammamieh*, Nabarun Chakraborty, Mohsen Barmada, Rina Das and Marti Jett (2005). Differential effects of omega-3 and omega-6 fatty acids on global gene expression in MDA-MB-231 breast cancer cell line. ASBMB, San Diego, CA April 2005.
i) Rasha Hammamieh*, Nabarun Chakraborty, Mohsen Barmada, Rina Das and Marti Jett (2005). Effects of omega-3 and omega-6 fatty acids on global gene expression in breast cancer cell lines. Era of Hope Breast Cancer Meeting, Philadelphia, June 2005.
j) Rina Das, Rasha Hammamieh, Jackie Madison, Nabarun Chakraborty, Agnes Day and Marti Jett (2005). Molecular Analysis of Differences in Breast Cancer in African American versus Caucasian Women. Era of Hope Breast Cancer Meeting, Philadelphia, June 2005.

Submitted abstracts for presentations:

a) Nabarun Chakraborty, Rasha Hammamieh, Agnes Day and Marti Jett. Differential effects of omega-3 fatty acids on global gene expression of breast cancer. Submitted to FASEB J. for ASBMB April 2006.

Collaborative Research Projects

The projects listed below were established and have resulted in published journal articles or accepted manuscripts.

- Dr. John Stubbs (HUCC) -Dr. Rina Das (WRAIR) . Enrichment of cells using sterile cell sorting technologies; Use of inducible vectors along with GFP vectors to study the effect of selected proteins on breast cancer in animal models. Cells have been transfected with the TET on vector, demonstrated with a GFP vector to show transfection is constitutive.
- Dr. John Stubbs (HUCC) –Dr Sachin Mani (WRAIR) Use of the IVIS imager to study the course of tumor development using African-American cells transfected with GFP in vivo using repeated imaging.
- Dr. Agnes Day, Jacqiline Mason (graduate student), Dr. Haile Yancy (post-doc), Kerrie Lashley (undergraduate) of HUCC and Marti Jett (WRAIR) have done *in vitro* microarray studies on metalloproteinase expression between African American and Caucasian cell lines and are currently exploring regulatory pathways involved in MMP expression.
- Dr Agnes Day and Dr. Robert Canada. Studies aimed at the induction of MDR in African-American cell lines and the use of resistant cells in our animal studies, especially looking for properties related to metastasis.
- Dr. Rina Das and Dr. Marti Jett (WRAIR)- Dr. Agnes Day (HUCC) Effect of fatty acids on the growth and gene expression of African American breast cancer cell lines. Two cell lines were grown in the presence of different fatty acids with tumor promoting and tumor inhibiting activity. RNA has been isolated and analyzed using Agilent Bioanalyzer chips. They will be tested on human gene chips for expression patterns, compared with Caucasian breast cancer cell lines.
- Dr. Rasha Hammamieh (WRAIR) – Dr. Agnes Day (HUCC) Microarray analysis of cancerous cell lines from African American vs. Caucasian cell lines of breast cancer cells. Four African American cell lines were obtained from ATCC were cultured and RNA isolated. Four Caucasian cell lines (age, ER status and menopausal stage matched) obtained from ATCC, cultured and RNA isolated. Staff members of Dr. Day's laboratory were trained to run microarray experiments using custom chips generated at WRAIR. The RNA samples were hybridized to human cDNA gene chips at WRAIR.
- Dr. Robert Canada of the Physiology & Biophysics Dept., and Agnes Day (HUCC). Comparative studies of Gallidinium drug-resistance in African American Breast Cancer cells. This project will compare with prior studies of such effects on Caucasian cells.
- Robert Canada, Agnes Day Rina Das and Marti Jett. In vivo imaging of African American cells transfected with luciferase to investigate anti breast cancer approaches most promising for these unusually aggressive African American Breast Cancer Cells.

Students Trained:

All of the graduate students/Fellows listed below have been supported-in-part by this training program. Most have participated in all of the workshops and all are involved in research projects involving cancer.

- Tamara Tatum-Broughton, Microbiology 6th year graduate student.
- Douglas F. White, Microbiology, 5th year graduate student.

- Jacquline A. Mason, Microbiology, 4th year graduate student.
- Kerry Lashley, Anatomy, 2nd year graduate student (formerly an undergraduate trainee).
- Dr. Haile F. Yancy, Post-doctoral Fellow, Microbiology.
- Dr. Nae'em Abdullah, Post- doctoral Fellow (1 year), currently 2nd year Pathology Fellow, Howard University College of Medicine and Howard University Hospital.
- Altriesha Foster, Microbiology, 2nd year graduate student.
- Sandra Dillahunt, Microbiology, 2nd year graduate student.
- Sharla Peters, Microbiology, 2nd year graduate student
- Charles Thompson, III, 3rd year Medical student
- Dr. Gay Morris, Biology, PH. D. recipient Dec. 2004. Now a post-doctoral associate.

Current Courses:

- Integrative Oncology. This is a multidisciplinary graduate level course, which was offered through the Microbiology department, Spring 2004. Eight students registered for the course (Microbiology, Psychology, Social Work, Nutrition and Genetics). Topics included cancer biology, genetics of cancer, viral vectors in cancer, oncogenes, hormonal regulation of cancer, and cancer epidemiology. Lecturers were from Howard University, Johns Hopkins University, Georgetown University, Walter Reed Army Institute for Research, and the National Cancer Institute. It was open to undergraduates, graduates, postdoctoral fellows, medical students, medical residents and faculty. There were at least 30 individuals present per lecture - post-docs, faculty, medical residents and physicians- indicating that this course was topical and long overdue.
- Molecular Biology. The current molecular biology course offered within the Department of Microbiology will be enhanced through the addition of didactic lectures and/or laboratories on the following subjects: Gene array analysis; real time PCR; DNA methylation analysis; prokaryotic and eukaryotic expression vectors; stable eukaryotic transformation and selection; and phage display analysis of protein-protein interactions.

Continuing Course

- Basic Oncology: Due to the varied background of the registrants in the Integrative Oncology Course, (Sociology, Psychology, Nutrition), we have created a basic oncology course so that non-biology majors will be introduced to the basic concepts of normal and diseased cells, nucleic acids, cell signaling, mutations, carcinogens and mechanisms of cancer induction. This course is currently in review for addition to the curriculum.
- Therapeutic strategies for breast cancer: Dissemination of a course of study designed for high school students from inner city DC public schools. Students carried out proliferation studies using mouse mammary gland cancer cells incubated with anticancer drugs. Discussions of differences in prevalence of disease highlighted the experiences of these students and their families.

Conclusions:

In so many cases, we have exceeded our aims outlined in the proposal's statement of work. The only aim still pending relates to the IVIS imager and that was a mechanical adaptation to the instrumentation (completed in August 05). We have many collaborative projects, have trained a new cohort of students destined to become the next generation of cancer researchers. The PI and other faculty have been successful at obtaining additional grant funding from a variety of sources. We are seeking further funding to continue and expand this collaboration. The data generated thus far have resulted in numerous published manuscripts, abstracts and presentations. One modest NCI U56 Pilot Project grant has enabled us to purchase specialized equipment required to continue these studies, and provide stipend support for additional graduate students involved in the research. The newly established collaborations should prove fruitful and generate additional publications and research grant proposals. New topics for established trainees (in vivo imaging, SCID mice with xenografts, Biostatistics and Experimental Design), and a continuation of the animal handling and mammary gland workshops will be performed or continued in the upcoming year should our no-cost extension be approved.

APPENDIX

Publications

Published full manuscripts

Manuscript #1.
Tesema, Y., Raghavan, D., and Stubbs III, J. (2005) Bone cell viability on methacrylic grafted and collagen immobilized porous poly (3-hydroxybuterate-co-3-hydroxyvalerate) J. Appl. Polymer Sci. 98:1916-1921 http://www3.interscience.wiley.com/cgi-bin/fulltext/112092854/PDFSTART
NOTE: This article sets up an artificial bone model and provides a potential metastasis model for specific breast cancer cell lines established from African American women.

Manuscript #2
R. Hammamieh, M. Anderson, K. Carr, C. Tran, D. Yourick and Marti Jett (2005). Students Investigating the Antiproliferative effects of synthesized drugs on mouse mammary tumor cells. Cell Biology Education 4:221-234.

Manuscript #3
R. Hammamieh, N. Chakraborty, M. Barmada, R. Das and Marti Jett (2005). Expression patterns of fatty acid binding proteins in breast cancer cells. J. Experimental Therapeutics and Oncology 5:133-143.

Manuscript Submitted--#4
L.Y. Berkeley, O.O. Kassim, A. Day, R. Kittles, W. Austin, L. Frederick, M. Coomes, J. Stubbs (2005) Phenotypic, Biochemical and Genetic Characterization of *Bacillus sp. BFAS* An Antifungal Producing Bacterium (Submitted to J. Canadian Microbiology). This extract may have anticancer activities for breast cancer.

Manuscript in Preparation--#5
Haile F Yancy[1,2], Sharla Peters[2], Charles E Thompson III[2], Jacquline A Mason[2], George K Littleton and[3] Agnes A Day*[2] Comparative Study of Gene Expression during Metastatic Progression between African American and Caucasian Women. In preparation for Cancer Research

NOTE: many abstracts/presentations have been published under this training grant and those items are available.

Bone Cell Viability on Methacrylic Acid Grafted and Collagen Immobilized Porous Poly(3-hydroxybutrate-*co*-3-hydroxyvalerate)

Y. Tesema,[1] D. Raghavan,[1] J. Stubbs III[2]

[1]*Polymer Group, Department of Chemistry, Howard University, Washington, District of Columbia 20059*
[2]*Department of Microbiology, College of Medicine, Howard University, Washington, District of Columbia 20059*

Received 22 September 2004; accepted 28 March 2005
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Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Porous poly(3-hydroxybutrate-*co*-3-hydroxyvalerate) (PHBV) film was prepared by solute leaching of salt/PHBV cast film. The surface chemistry of the PHBV membrane was modified by performing graft polymerization of methacrylic acid (MAA) on ozone treated porous PHBV film, followed by immobilization of type I collagen. The surface characteristics of the modified and nonmodified porous films were measured by water contact angle. The rat osteosarcoma cell line UMR-106 osteoblast like cells were used as model cells to evaluate the cell viability on surfaces. The initial cell attachment, growth pattern, and proliferation as measured by MTT assay were used to evaluate the bone cell viability on the modified and nonmodified films. Among the PHBV films studied, the nonmodified porous PHBV and the porous PHBV film type I collagen dip coated showed no significant difference in cell attachment and proliferation, while the porous PHBV membrane that was collagen immobilized after MAA grafting showed considerable activity of osteoblast like cells. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 98: 1916–1921, 2005

Key words: biocompatibility; chemical grafting; osteoblast cell activity; porous polymeric membrane

INTRODUCTION

Biodegradable polymers have gained considerable importance in the biomedical industry as scaffold and in the development of biomaterials for therapeutic applications. In particular, poly(3-hydroxybutrate-*co*-3-hydroxyvalerate) (PHBV) has received enormous attention for biomedical application in part due to the ability to tune the characteristics of PHBV to achieve desired mechanical properties and rate of degradation.[1] Since PHBV is relatively hydrophobic, the low surface energy of the PHBV affects cell attachment and growth.[2–4]

For a given environment, the cellular interaction with a polymer is strongly dependent on the surface characteristics (i.e., topography and chemistry) of the polymer.[5–8] Polymer surface chemistry, especially the wettability of the surface, has been shown to influence the initial cell attachment through the adsorption of proteins derived from serum used in the culture medium. In the absence of natural recognition sites of cells on the surface of PHBV, commonly surface treatment techniques are used to functionalize the polymer surface so as to promote favorable cellular and physiological response. The cell attachment is believed to involve the adhesion receptors on the cell surface and extracellular matrix (ECM) proteins adsorbed to the polymer surface. To mimic the natural environment on polymeric surfaces, the treated polymeric surfaces have been modified by coating or grafting extracellular matrix (ECM) proteins (fibronectin, vitronectin, collagen) that have a cell-binding domain containing the RGD sequence.[9–14]

The anchoring of extracellular matrix protein to the polymer surface and its influence on osteoblastic cell attachment and growth was a part of our recent investigation. A collagen coated surface can perform significantly different from the collagen anchored surface because collagen can be removed from the dip coated surface when exposed to culture medium with time. Our preliminary results suggest that in a favorable biological environment, collagen chemically immobilized on PHBV is a better material for supporting bone cell growth than collagen dip coated PHBV and untreated PHBV.[15]

In addition to our interest in understanding the role of the immobilization of proteins in supporting osteoblastic cell proliferation on a polymer surface, we consider it pertinent to examine the combined effect of porosity and immobilization of proteins in cell attachment and growth for the development of tissue engineered medical products. High porosity and high interconnectivity of pores are required to minimize the

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amount of polymer usage, to increase the amount of surface area available for cell anchoring, and to assist in the transport of nutrient and cellular byproducts across the porous membrane.[16]

Several methods have been developed to prepare highly porous scaffolds. They include phase inversion techniques such as liquid-liquid and liquid-solid phase separation,[17] leaching of the soluble phase from the multiphase polymer blend, or leaching of soluble particulates from particulate composite.[18,19] For achieving complete removal of the particulate, the particulate fraction should exceed the percolation threshold (about 40 wt %) so that a number of significant pathways for the diffusion of the leaching solvent are generated. In recent studies, we showed that when particle concentration in the composite exceeds the percolation threshold, with the exception of very few particles in the bulk of the film, the particle leaching in the composite is very high.[20,21] Depending on the particle cluster size and the film thickness, one can obtain localized percolation pathways, which is an interesting thin-film effect of porous material.

To further enhance the pore structure and pore interconnectivity of the porous biomaterial, several studies have tried to combine particulate leaching with freeze-drying, gas foaming, and solvent casting.[22–24] Among these various methods, solvent casting in combination with particle leaching was chosen to fabricate three-dimensional porous scaffold.[25] The objective of the study was to address whether porous film with collagen chemically immobilized or physically immobilized impact osteoblastic cell attachment and growth. In this study, confocal microscopy was used to map the cell infiltration after 5 days on modified and unmodified porous PHBV film.

METHODS

Materials

Poly(3-hydroxybutrate-*co*-3-hydroxyvalerate) containng 8 wt % hydroxyvalerate (PHBV), type I collagen (calf skin), methacrylic acid (MAA), 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and Bradford reagent were purchased from Sigma Aldrich (St. Louis, MO). MAA was purified by distillation under reduced pressure prior to use for the grafting experiment. 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) were obtained from Fisher Scientific. Rat osteosarcoma cell line UMR-106 (ATCC CRL-1661) was purchased from the America Type Culture Collection.

Membrane preparation

Porous PHBV membranes were prepared by using a combination of solvent casting followed by solute leaching techniques. Sieved sodium chloride particles of size 75–150 μm (1.5 g) were hand mixed with 1.0 g of PHBV powder and dissolved in 10 mL of $CHCl_3$ at 60°C. The resulting dispersion was cast in a 9 cm petri dish, and the solvent was allowed to gradually evaporate over 24 h by covering the petri dish partially with a lid. The resulting PHBV/NaCl composite membrane was conditioned for 1 day at 24 ± 2°C under vacuum before further use. The membrane was immersed in 100 mL of distilled water in a shaker at 25°C for 2–5 days (the water was changed every 2 h for the first 12 h, then 2–3 times a day) to leach out the salt. The salt-free PHBV membrane was air and vacuum dried for 24 h and stored in a dessicator until further use. The drying process was continued until constant weight of the film could be obtained.

Ozone treatment and PMAA grafting of porous PHBV film

Details about ozone activation and grafting of MAA of porous PHBV film can be found elsewhere.[15] Briefly, a rectangular piece of membrane 2 × 4 cm^2 of thickness 1.2 mm was flushed with air containing 2.2 g/h ozone generated using an ozone generator (Ozonology, Model # L-25, Evanston, IL) for a predetermined time interval. The chamber was then purged with oxygen for 10 min to remove unreacted ozone. Contact angle measurements of activated and untreated porous PHBV film were collected in triplicate.

The activated membrane was retrieved and placed in a Pyrex glass tube that contained 5 wt % methacrylic acid (MAA), 0.2*M* H_2SO_4, and 1mM $FeSO_4$. The grafting experiment was performed at 65°C for 1 h in a nitrogen environment, and the grafted membrane was retrieved and rinsed with double-distilled water to free it of residual MAA. The amount of carboxyl groups grafted onto porous PHBV membranes was determined by simple acid base titration using a literature procedure.[26]

Collagen immobilization on PMAA grafted PHBV membranes

MAA grafted porous PHBV membranes were cut into discs of 10 mm diameter and placed in a solution (containing 10 mg/mL of EDAC in a phosphate buffer solution (PBS) maintained at pH 4.5) for 24 h at 2–4°C. The EDAC treated membrane was then placed in a solution of type I collagen (4 mg/mL in 0.3% acetic acid) for 24 h at 2–4°C. The collagen-grafted membrane was retrieved and sonicated with double-distilled water to free the membrane of physisorbed collagen. In addition to preparing collagen grafted PHBV membrane, collagen physisorbed PHBV film was prepared by dip coating untreated porous PHBV film into a 4 mg/mL collagen solution (prepared in 0.3% acetic

acid) for 24 h. The collagen density on the individual PHBV membranes was determined as described by Shu et al.[27] and the Bradford method.[28]

Culture and preparation of cells for cell attachment and proliferation studies

UMR-106 cells were cultured in 0.22 μm filtered Dulbecco's modified essential medium (DMEM) supplemented with 10% (w/v) heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL (w/v) streptomycin (Quality Biological, Inc.), and 50 μg/mL L-ascorbic acid (Fisher Scientific) at 37°C in 5% CO_2. At termination, cells were harvested and washed with Hank's balanced salt solution (Sigma Aldrich) and trypsinized with Trypsin-EDTA (0.05% trypsin, 0.1% EDTA) (Quality Biological, Inc.) for 15 min to obtain a cell suspension. Trypsin activity was inhibited upon the addition of FBS at a final concentration of 10%. Next, cells were centrifuged and washed with 0.45 μM filtered sterilized serum-free DMEM three times to remove residual FBS. The concentration of the resulting cell suspension was determined with the use of a hemocytometer. Care was taken during the culture preparation and viability studies to use sterile techniques.

To asses cellular attachment and proliferation on the PHBV membranes, cells were seeded at 3×10^4 cells/cm^2 and 2×10^4 cells/cm^2, respectively, in 96 well tissue culture plates in 200 μL serum-free DMEM. Cells were also seeded on tissue culture polystyrene (TCPS) at identical conditions, a positive control for the study. The cells were incubated for 1, 3, and 5 h at 37°C in 5% CO_2. For the cell attachment study, the film was retrieved from the well after 1, 3, and 5 h incubation and washed with serum free DMEM and the number of adhered cells to the film was determined by MTT assay.[29] For the cell proliferation study, the film was retrieved from the well after 24 h incubation, washed with serum free DMEM, and resuspended in 10% serum containing DMEM media. After 5 days, the films were collected and washed with serum free DMEM and MTT assay was performed.

Briefly, 100μL of MTT (5 mg/mL) was added to each culture well containing 200 μL serum free DMEM, and the membrane was allowed to incubate for 3 h at 37°C with 5% CO_2. During incubation, the viable cells are involved in the mitochondrial reduction of MTT to a dark blue formazan product. Following incubation, the MTT solution was removed and the membranes were rinsed twice in PBS. The water insoluble formazan product was dissolved by adding 200 μL of dimethyl sulfoxide (DMSO) and transferred to a 96 well plate. The absorbance was measured at 595 nm using a Titertek Multiskan 310 C plate reader.

Figure 1 Optical micrograph of porous PHBV film after salt leaching by deionized water over 5 days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Confocal microscopy characterization

Confocal microscopy and 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) were used to visualize viable adherent cells within the porous membrane. Cellular esterases in viable cells convert the non fluorescent 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) into the fluorescent 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein BCECF compound.[30–32] Briefly, viable cells were stained by incubating the membranes containing cells with 5 mg/mL BCECF-AM in serum-free media for 1 h followed by rinsing in PBS. The membranes were examined using an upright laser confocal microscope (Olympus). The key feature of confocal microscopy is that only light from a narrow objective focal plane is detected. Intracellular BCECF was excited at 488 nm using an Ar+ laser light, and light emitted (505–550 nm) from the film was detected with a photomultiplier tube. The 10× objective was used for the study. Images were acquired by focusing the laser beam at 300 μm beneath the surface of the film.

RESULTS AND DISCUSSION

Porous PHBV film was prepared by solvent casting of a salt : PHBV solution followed by solute leaching over a duration of 2 to 5 days. Figure 1 shows images of PHBV film that were prepared with pores having a characteristic dimension in excess of 75 μm with a maximum of 150 μm (based on optical micrograph data). The micropores formed were cuboids and were dispersed throughout the film. The size and shape of the pores are in general agreement with the salt particle dimension used for the study. Although we restricted the porous membrane preparation to a single

TABLE I
Amount of Carboxyl Group Introduced After Grafting, Amounts of Collagen Immobilized, and Water Contact Angles on Porous PHBV Surfaces

	-COOH introduced (nmol/cm^2)	Collagen density (μg/cm^2)	Water contact angle (°)
PHBV	—	—	65 ± 1.3
PHBV-g-PMAA	0.08 ± 0.06	—	52 ± 2.1
PHBV-g-PMAA-COLL	—	3.4 ± 0.91	54 ± 0.9
PHBV-COLL	—	1.1 ± 0.87	55 ± 1.4

salt composition, in a related study we have shown that interconnectivity of the pores is strongly related to the particulate composition and particle size range. For example, when the leachable component exceeds 40 wt % of the overall composition of the composite, 95 wt % of leachable component in the particulate composite is leached, leaving behind pores in the composite, because of the well established interconnecting percolation pathways for solvents to access the leachable component.[20,21] For this study, we limited our work to porous membranes prepared from 60 wt % salt : 40 wt % PHBV film.

In our earlier publication, we described the procedure for grafting collagen on PHBV film.[15] Similarly, microporous PHBV membranes were ozone treated and MAA grafted followed by collagen chemical immobilization. The water contact angle data of the individual membranes is shown in Table I. Water contact angle values of the membranes were measured by the sessile drop method. As expected, the contact angle of MAA grafted PHBV membranes was significantly lower (more hydrophilic) than the unmodified PHBV film. A word of caution is in order that measuring the contact angle of porous material is not an accurate reflection of the hydrophilicity of the membrane because of the irregularity of the film. However, these results follow the trend noticed in PHBV film, where the contact angle of PHBV film was shown to decrease with exposure of PHBV film to ozone, grafting of MAA, and collagen immobilization. The exposure of the porous PHBV membrane to ozone gives rise to the formation of a variety of functional groups, including peroxides and hydroperoxides.

To establish that indeed polar functional groups (as evidenced by water contact angle data) are formed during ozone activation, the amount of collagen present on dip coated and chemically immobilized membranes were compared. As mentioned in Table I, the collagen density on collagen immobilized membrane was found to be higher than that of collagen dip coated membrane. The PMAA grafted membrane provided anchoring sites for collagen immobilization, because of the strong interaction of negative charge of the carboxylate group of MAA grafted chains with the cationic groups of the collagen molecule.[33,34]

Washing the membranes resulted in some loss of the collagen molecules from the grafted porous PHBV membrane (about 15%), and significant loss (about 50%) of the collagen molecules from the dip coated porous PHBV membrane. PMMA grafting of the PHBV membrane surface was beneficial in strongly binding collagen molecules to the film surface.

The membranes that were seeded with cells were used in the measurement of cell attachment, cell proliferation, and infiltration of cells into the porous film network structure. To assess cell viability on the porous membranes, cell density was assayed by the MTT optical density assay, and the cell morphology on the PHBV membrane was mapped using a vital fluorescence microscopy technique. Figure 2 shows UMR-106 osteoblastic cell attachment data on various membranes relative to incubation time. Cell attachment is expressed as a ratio of cells attached on a specific membrane relative to tissue culture polystyrene (TCPS) by performing cellular measurements under identical conditions. The results are an average of multiple measurements for all three sample types. It must be mentioned that the cell adhesion studies were performed in serum free media to eliminate the contribution of other proteins in the cell culture media to the number of cells adhered to the porous film. The rate of cell attachment on each of the three membranes was very similar. There is a gradual increase in the number of cells attached to the dip coated PHBV-COLL porous membrane or the chemically modified PHBV-g-PMMA-COLL porous membrane. However, the greatest number of attached cells was observed on the collagen immobilized PHBV porous membrane relative to the collagen dip coated PHBV or the nonmodified PHBV porous membrane.

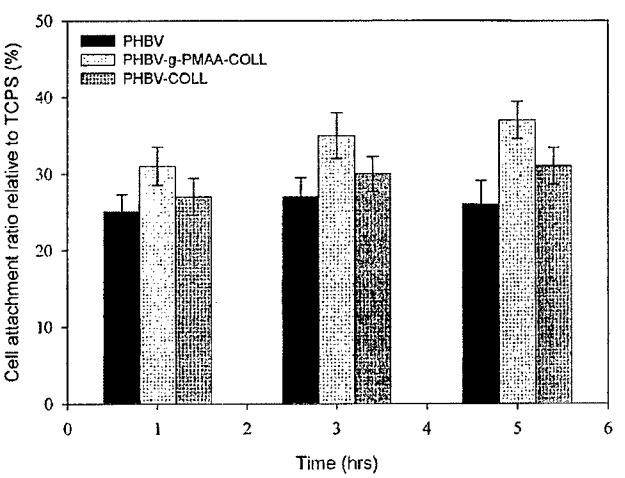

Figure 2 Cell attachment ratio of porous PHBV, collagen dip coated PHBV, and COLL-PMMA-g-PHBV membrane relative to TCPS.

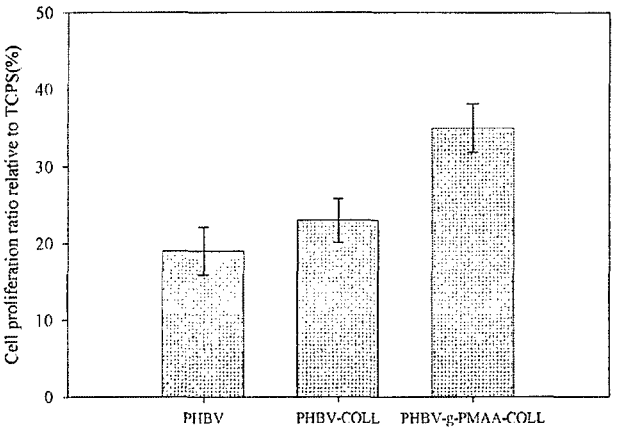

Figure 3 Cell proliferation ratio of porous PHBV, collagen dip coated PHBV, and COLL-PMMA-g-PHBV membrane relative to TCPS.

Figure 3 provides the cell proliferation data on the individual membrane types. The number of cells attached after 24 h effectively formed the nucleation point for further cell proliferation study, since any nonadherent cells in the attachment assay were removed via washing prior to the transfer of the membrane to a serum containing a culture medium that facilitates growth. Chemically grafted COLL-PMMA-g-PHBV porous membranes exhibited the highest cell proliferation potential when compared to the collagen dip coated PHBV or the nonmodified PHBV porous membrane films. Cells on untreated porous PHBV membrane exhibited ~ 50% of the cell proliferation observed on the collagen immobilized porous membrane.

The fluorescence images in Figure 4 show UMR-106 cells on porous PHBV film at 300 μm beneath the surface. Osteoblast cells seem to have proliferated the various membranes during incubation. Abundant viable cells (as observed by a high percentage of the cells exhibiting fluorescence intensity) were observed for the collagen immobilized membrane when compared to either the collagen dip coated or the unmodified porous PHBV membranes. From the confocal microscopy characterization results and MTT assay, it seems that the porous PHBV membrane after PMAA grafting and collagen immobilization provides a favorable surface for UMR-106 cell attachment and subsequent proliferation.

CONCLUSIONS

The following conclusions can be drawn from the current work:

1. Porous PHBV membranes were prepared by the conventional salt leaching technique. The

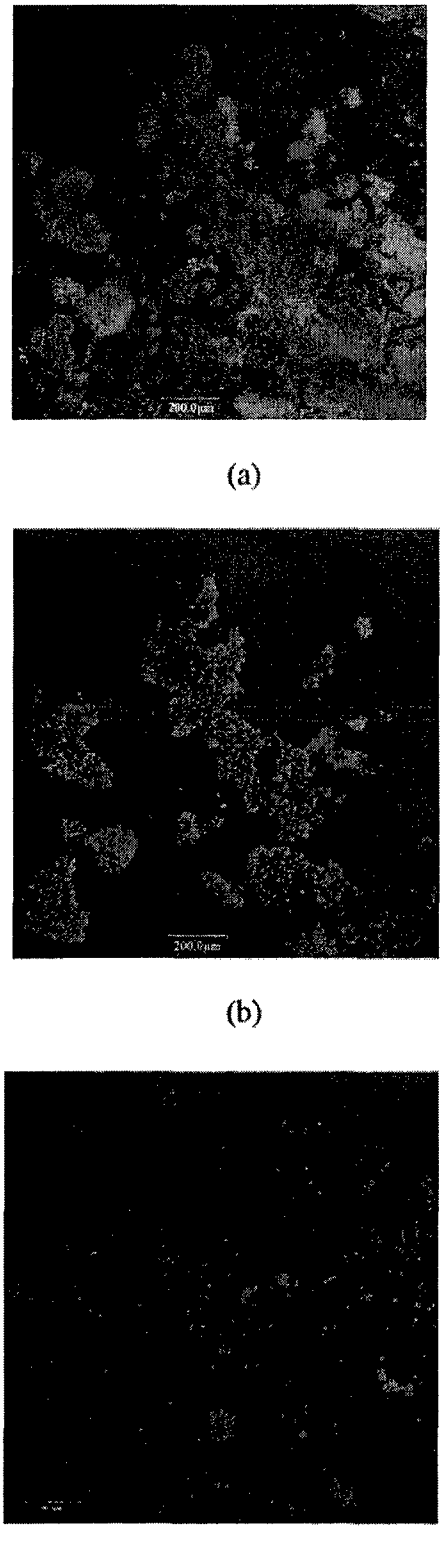

Figure 4 Confocal micrograph of 3 day incubated: (a) COLL-PMMA-g-PHBV, (b) collagen dip coated PHBV, and (c) porous PHBV membrane. Micrographs taken at 300μm beneath the surface. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

membranes were dip coated or chemically grafted with collagen. The chemically grafted PHBV membrane had a higher density of collagen than the dip coated PHBV membrane.
2. Collagen immobilized PHBV membrane provided a more favorable matrix for cell proliferation than either dip coated collagen PHBV porous membranes or unmodified PHBV porous membranes.
3. Confocal microscopy imaging is a valuable tool to map viable cells buried in the porous PHBV matrix.

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Article

Students Investigating the Antiproliferative Effects of Synthesized Drugs on Mouse Mammary Tumor Cells

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The potential for personalized cancer management has long intrigued experienced researchers as well as the naïve student intern. Personalized cancer treatments based on a tumor's genetic profile are now feasible and can reveal both the cells' susceptibility and resistance to chemotherapeutic agents. In a weeklong laboratory investigation that mirrors current cancer research, undergraduate and advanced high school students determine the efficacy of common pharmacological agents through in vitro testing. Using mouse mammary tumor cell cultures treated with "unknown" drugs historically recommended for breast cancer treatment, students are introduced to common molecular biology techniques from in vitro cell culture to fluorescence microscopy. Student understanding is assessed through laboratory reports and the successful identification of the unknown drug. The sequence of doing the experiment, applying logic, and constructing a hypothesis gives the students time to discover the rationale behind the cellular drug resistance assay. The breast cancer experiment has been field tested during the past 5 yr with more than 200 precollege/undergraduate interns through the Gains in the Education of Mathematics and Science program hosted by the Walter Reed Army Institute of Research.

Keywords: undergraduate, secondary, mouse tumor cells, breast cancer, personalized cancer management, genomic profiling, cellular drug resistance assay

INTRODUCTION

The Walter Reed Army Institute of Research (WRAIR) recognizes the necessity of engaging, channeling, and maintaining student participation in the quantitative disciplines. Its strong commitment to education is evidenced by the Gains in the Education of Science and Mathematics (GEMS) program that provides precollege students the opportunity to participate in laboratory research. Over the past 5 yr almost 1,000 precollege interns have received a minimum of 40 h of laboratory training from WRAIR scientists. Each year, the WRAIR extends its outreach to the undergraduate population by accepting approximately 100 undergraduates as valued research assistants. To address the needs of this diverse student population within an active research facility while still adhering to strict deadlines and research standards, the WRAIR has developed several best practices that educate, review, and assess basic laboratory skills. These experiments are adapted from current in-house research and are interdisciplinary in that each incorporates chemistry, mathematics, biology, cell biology, and scientific writing as part of the protocol.

The breast cancer experiment was designed to be a concise, 5-d independent project. For most undergraduates, this study becomes their first experience with a multiday experiment and its attendant accountability. In our facility, mentors have used the breast cancer assay as a rapid assessment of the undergraduate's overall abilities. For the high school student, there is the excitement of discovery and working with actual cells. The undergraduates appreciate its summation of common cell biology techniques, and the younger teens enjoy generating their own data and solving the mystery.

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The modified antiproliferative drug assay was selected as an experimental model because it is broadly applicable (Anonymous, 1999). In addition, it engages students with its robust response of dead or live cells. Student researchers use in vitro mouse mammary tumor (MMT) cultures and common chemotherapeutic drugs for the assay. Three common cancer regimens—antioxidant, anti-inflammatory, and antimitotic—are tested (Krzystyniak, 2002; Lou and Chen, 2003). Over-the-counter drugs such as aspirin are often included to demonstrate ineffectual therapies. Spectrophotometer readings provide students with an instrumental and mathematical measure of cell viability. This 5-d immersion in cell biology experimentation delivers the excitement of discovery in a cogent framework that integrates multiple technical skills. Furthermore, it requires students to synthesize content knowledge into a single effort.

BACKGROUND FOR INSTRUCTOR

Breast cancer remains the most frequently diagnosed nonskin cancer in women. Even though 5-yr relative survival rates have been steadily increasing since 1974 from 75% to 85%, breast cancer is still expected to claim 40,410 lives in the United States (National Breast Cancer Foundation, 2005; National Cancer Institute, 2005) and 1.2 million lives worldwide (Imaginis: The Breast Health Resource, 2005). An estimated 211,240 new cases of invasive breast cancer and another 58,490 new cases of in situ breast cancer will be diagnosed by the end of 2005 in the United States. These numbers remain high despite advances in our understanding of cancer's etiology, pathology, and clinical management. Fortunately, the recent emergence of genomic technologies holds therapeutic potential for personalized cancer management.

Personalized cancer management merges standard chemotherapy and radiation treatments with genomic profiling and in vitro cell proliferation studies. Individualized genomic profiling allows the researcher to identify specific genes that contribute to unregulated cellular proliferation as well as those genes that inhibit the apoptotic mechanisms that normally control cellular growth (Evan and Vousden, 2001; Lou and Chen, 2003; Radice, 2002). By determining the molecular profile of a particular cancer, rational treatment regimens can be designed that focus on those gene products that are either hyperactive or inactive in a particular tumor. A patient's genome can also reveal susceptibility to certain toxic effects of chemotherapeutic agents (Strauss, 1999).

MATERIALS AND METHODS

The Laboratory Schedule

The cellular drug resistance assay experiments are recommended for undergraduate students entering the biological sciences or allied health laboratory or for use in an advanced high school biology course. This weeklong study introduces student researchers to a relevant scientific problem and provides them with a means of solving that problem using techniques common to all laboratories. The level of difficulty is adjusted by the number of appendices/preparations that are completed by the instructor rather than the student. All experiments were conducted in laboratories at the Walter Reed Army Institute of Research.

Below are brief summaries of each day's expectations. Appendices A through C provide detailed instructions for instructors or advanced students. Student handouts for days 1 through 5 (Appendix D) consist of detailed protocols and questions that guide student activity and thinking. It is assumed that the instructor will maintain sufficient cultures and drug stocks and has introduced students to basic techniques and skills.

Day 1. Day 1 begins with a brief interactive discussion or review about the origin and evolution of cancer. As newly christened research assistants, the students then receive their daily outlines and are referred to the laboratory supplies table and handed a conical tube of MMT cell culture. The student/team then prepares two 96-well plates and incubates the cells overnight.

Day 2. Students confirm cell viability and confluence. All students complete a Drug Assay Worksheet (Appendix E) that is verified by the instructor. The student/team receives two samples of an unknown cancer treatment drug or combination of drugs. The completed Drug Assay Worksheet becomes the individualized protocol for that numbered drug(s). After preparing and diluting the original stock solution, students add the various concentrations of drugs to their plates. Cells are incubated with the drugs for 48 h.

Day 3. At the beginning of day 3, the class is given an aliquot of MMT cells. Referring to a worksheet that outlines the steps of using the hemocytometer, the student learns to do a cell count and dilution. Day 3 is also a day for further discussion and beginning the report.

Day 4. In preparation for data from the spectrophotometer, the students learn to analyze sample figures, create plots, and carry out simple statistical analyses on sample data using both Microsoft Excel software and the statistical analysis features of the TI-83 graphing calculator. Cells complete the second incubation day and are placed in a freezer overnight.

Day 5. Students treat each sample well with CyQUANT lysis buffer and the fluorescent dye. The plates are scanned with a Multifluor FX scanner, and students analyze the data by taking the average intensity for each concentration of the drug used in each treatment and comparing it with the control wells. After analyzing their data, students contribute their information for group discussion and their drugs are revealed.

Assessment. For undergraduates, a written laboratory report with a level-appropriate rubric is designed by the senior scientist. Precollege students are required to submit their final plots, which are then compared with standards. Many precollege students also complete a laboratory report and/or a poster. It should be noted that the two undergraduate authors on this report began their research while in high school. The essentials of how assessment is conducted within a military facility are detailed below.

Mouse Mammary Tumor Cell Culture and Maintenance

MMT 060562 cells and all cell culture media and reagents were purchased from the American Type Culture Collection

(ATCC; Manassas, VA). The MMT cells were maintained according to the manufacturer's instructions in Eagle's Minimal Essential medium that was modified by ATCC to contain 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1.5 g/L sodium bicarbonate. The medium was supplemented with 10% fetal bovine serum and 1% Pen-Strep (10,000 units/mL penicillin and 100 μg/mL streptomycin). Cell culture flasks were maintained in a humidified 5% CO_2 incubator at 37°C. Cell viability was regularly checked with an inverted microscope. The cells were subcultured at a 1:2 ratio as needed, and media were changed every other day (Figure 1, Appendix A).

The optimal cell concentration for plating was 5×10^4 cells/mL. Cell concentration was determined with 0.4% Trypan blue solution and a hemocytometer (Appendix B). Cells were plated at 200 μL/well in 96-well plates and allowed to reach ~70% confluence after 24 h. The MMT cells were then treated with varying concentrations of several therapeutic agents (or combinations of agents): curcumin, nordihydroguaiaretic acid (NDGA), tamoxifen, tamoxifen and curcumin, and tamoxifen and NDGA (Appendix C). Control and treated cells were incubated for 48 h at 37°C and 5% CO_2. (Secondary students who ask to test an over-the-counter drug or an herbal remedy can request an additional plate and develop their own dilutions/concentrations. These results become a science fair project.)

Cellular Proliferation Assay

Following a 48-h incubation period, the 96-well plates containing the drug-treated cancer cells were removed from the incubator. All media were removed, and the plates were frozen for at least 30 min at –80°C or overnight in a –20°C or –4°C freezer. Students prepared reagents from the CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

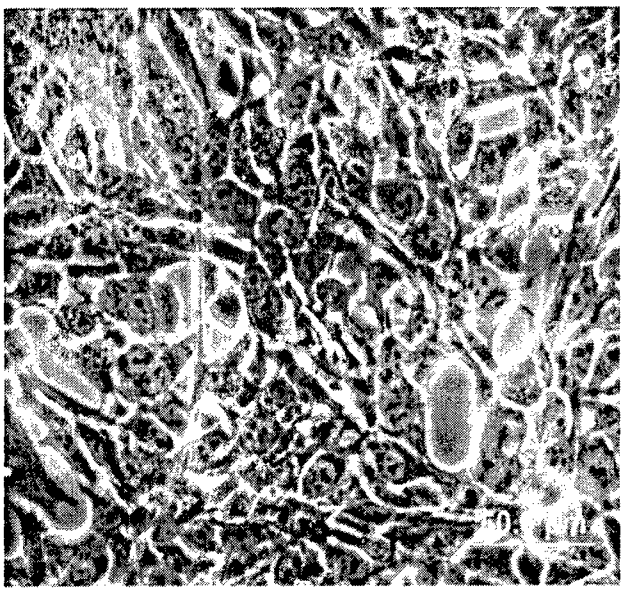


Figure 1. Photograph of MMT cells. Cells are grown to a confluent monolayer after a 24-h incubation at an initial concentration of 10^4 cells/well in modified Eagle's Minimal Essential medium.

The CyQUANT kit was selected as a convenient and sensitive procedure for determining the density of cells in culture. Cells were simply thawed and lysed by addition of a buffer containing the CyQUANT GR dye to each well (Anonymous, 1999). A green fluorescent dye, CyQUANT GR exhibits stronger fluorescent characteristics upon binding to cellular nucleic acids. The degree of fluorescence is then determined by scanning the plates with BioRad's Multifluor FX scanner and analyzing the images with QuantityOne software (BioRad, Hercules, CA).

During analysis, students convert pixel values corresponding to the scanned image of each sample well to numerical values that represent the adjusted volume (cells/mm^2) of cells in each sample. Standard school calculators are used to give the student researchers an introduction to research mathematics beginning with basic algebra. The intern then learns/reviews how to use a spreadsheet to analyze data exported from the scanner and how to create plots of cell density versus various concentrations of their unknown drug or combination of drugs compared with untreated controls.

Antiproliferative Therapeutic Agents for Treatment of Breast Cancer

Tamoxifen is an antimitotic drug that has been used against advanced breast cancer in postmenopausal, hormone-sensitive patients for over 20 yr (Grandishar, 2004). For hormone-sensitive neoplasms, the mix of estrogen receptor subtypes, or isoforms, is slightly modified to allow maximal estrogen binding and thus maximal stimulus for cellular proliferation. Although tamoxifen remains a successful antiestrogen therapy, recent research has revealed some serious shortcomings. First, it is now known that approximately 25% of breast cancers are not estrogen stimulated, making tamoxifen inconsequential. Second, only half of estrogen-sensitive cancers successfully respond to tamoxifen because of induced tamoxifen resistance by the enzyme protein kinase A (Chagpar, 2004). As a phytoestrogen, a traditional drug with well-documented effects, and a well-known hormonal antagonist, tamoxifen was selected as a test drug suitable for students.

Curcumin, a yellow compound from the turmeric root, is found in various Asian and Middle Eastern herbal remedies and cuisines. Its inhibitory effect on growth has been shown to be time- and dose-dependent and is correlated with the inhibition of ornithine decarboxylase (ODC) activity (Mehta and Pantazis, 1997). Interestingly, the enzyme ODC is used to manufacture a class of proteins, polyamines, that cause cancer cells to grow more rapidly. Curcumin also exhibits a modest ability to arrest cancerous cells in the G2S phase of the cell cycle and suppress c-jun/AP-1 and NF-kappa B activation pathways, and it possesses limited anti-inflammatory activity. Patients often use curcumin as a nutritional adjuvant to standard chemotherapy (Wallace, 2002) because even limited suppression of inflammatory eicosanoids appears to decrease the growth of breast carcinomas (Krzystyniak, 2002; Teh and Hill, 2004).

NDGA, commonly found in chaparral tea, is a constituent of the creosote bush *Larrea divaricata*. Historically, it has been used in Native American remedies for cramping, joint pain, allergies, parasites, and cancer (McGuffin and Hobbs, 1997). As a powerful antioxidant, NDGA was investigated as a possible cancer treatment. In vitro research has shown that NDGA acts as a selective inhibitor of lipoxygenases (LOX),

platelet-derived growth factor receptors, and protein kinase C intracellular signaling compounds. Each of these protein families plays an important role in the proliferation and survival of cancer. Current research has focused on finding more potent analogs of NDGA to ameliorate the high concentrations of the compound that are required for therapeutic efficacy (McDonald and Bunjobpon, 2001; Sheikh and Philen, 1997; Tong and Ding, 2002).

The activity of the antiestrogen agent, tamoxifen, was tested both individually and in combination with curcumin and NDGA. Because both curcumin and NDGA target disparate classes of proteins, it was hoped that the activity of tamoxifen would be augmented by the use of these supplementary agents. Curcumin and NDGA have historically been used as unconventional, alternative treatments for breast cancer.

RESULTS

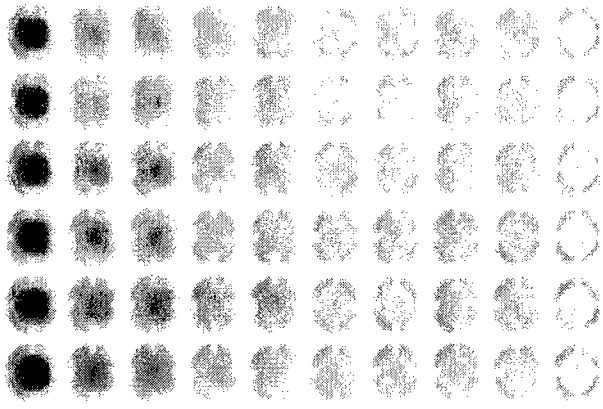
Cellular Proliferation of MMT Cells Exposed to Individual Drugs

The three drugs chosen for our study—curcumin, NDGA, and tamoxifen—each represent unique approaches to hindering abnormal cellular proliferation (Evan and Vousden, 2001; Strauss, 1999). Although they target disparate cellular processes implicated in the deregulation of cell growth, each drug individually caused decreases in the cell population in vitro.

Of the three, tamoxifen appeared to be the most potent treatment. Figure 2 shows the culture plate for MMT cells treated with tamoxifen at increasing concentrations from left to right. Tamoxifen induced a significant inhibition in cell growth of more than 85% compared with the untreated cells (Figure 3A). These high reductions in cell proliferation show no indication of tamoxifen resistance, as would be expected in a short, 2-d incubation. In vitro conditions cannot accurately simulate in vivo estrogen availability or physiological response to prolonged tamoxifen therapy. Longer incubation periods under in vivo estrogen levels might allow time for the cells to develop the compensatory mutations and alterations in the estrogen receptor composition and pathways.

NDGA treatment resulted in the least dramatic reduction, with proliferation relative to the control populations decreased by 20% (Figure 3B). The students considered two sources of error. The students considered 1) testing more concentrated doses of NDGA or 2) increasing the length of time the cells were exposed to the less concentrated doses.

A



B

Tamoxifen: Plate 1; adjusted volume (cells/mm^2)										
[mM]	**media**	**0.005**	**0.005**	**0.01**	**0.01**	**0.02**	**0.02**	**0.04**	**0.04**	**unkn**
well 1	20360.	4880.7	7249.0	5948.1	5803.3	9402.3	2277.3	6537.0	6594.0	14164.
well 2	21654.	7630.7	11019.	6602.4	4954.6	1960.1	14387.	4495.7	6819.2	3478.2
well 3	20732.	7038.6	9374.0	6253.2	4571.5	1490.1	12982.	10472.	6734.8	7791.7
well 4	18058.	6447.7	6199.4	6258.1	4714.3	1455.9	11585.	8180.1	7475.8	3778.4
well 5	18438.	8407.9	8653.0	5184.6	5580.5	1854.3	8468.8	11409.	1973.5	7561.1
well 6	17718.	7266.2	5700.0	5306.2	4762.0	14383.	6793.3	625.26	2206.8	7584.1
Avera	**19493.**	**6945.3**	**8032.5**	**5925.4**	**5064.4**	**5091.0**	**9415.9**	**6953.3**	**5300.7**	**7393.0**

Figure 2. (A) Picture of the culture plate for MMT breast cancer cells treated with tamoxifen at increasing concentration, from left to right. Fluorescence measurements were made with a microplate reader with excitation at 480 ± 10 nm and emission detection at 520 ± 12.5 nm. This figure shows fluorescence intensity decreasing as the concentration of tamoxifen increases. (B) Readings from one high school student's plate (not related to A). Note that the student did not put media in the second column, and he was not sure what he did for the final column. Learning to take comprehensive laboratory notes is part of the training and one reason students pool their data for the final plots.

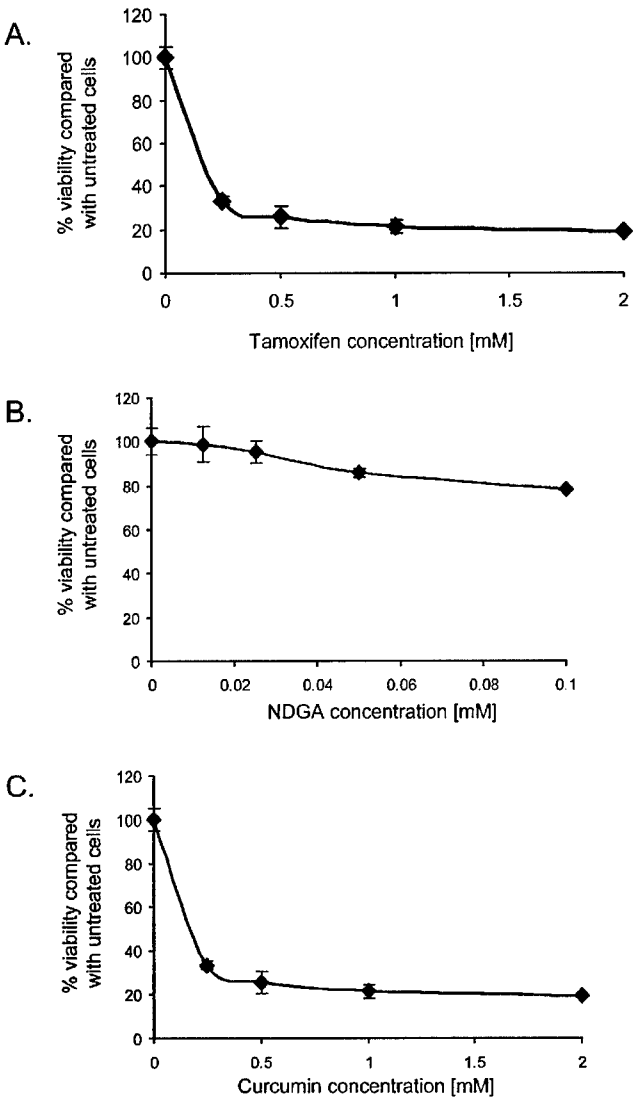


Figure 3. Concentration-dependent effects of (A) the antiestrogen tamoxifen ($n = 16$) on proliferation of MMT cells. (B) The LOX inhibitor NDGA ($n = 21$), and (C) the ornithine decarboxylase inhibitor curcumin ($n = 15$) after 48 h. Results are expressed as a percentage of control, as compiled from separate trials done by high school students.

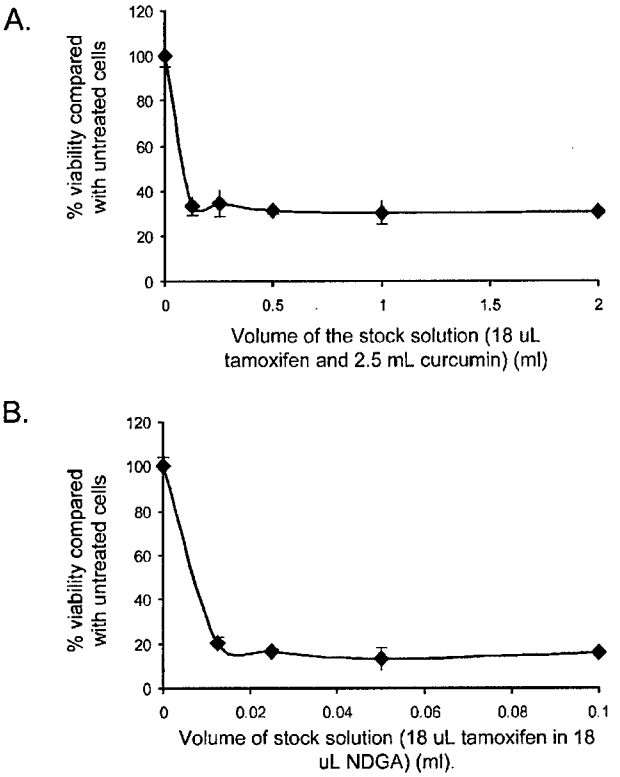


Figure 4. Concentration-dependent effects of (A) combination of tamoxifen and curcumin ($n = 22$) and (B) combination of tamoxifen and NDGA ($n = 13$) on proliferation of MMT cells after 48 h. See Appendix C for concentrations of stock solutions. Results are expressed as a percentage of control, as compiled from separate trials done by high school students.

The NDGA results are consistent with the mixed endorsement of the plant derivative as a dependable supplement to current breast cancer treatments. Oncologists remain hesitant to recommend the high doses of NDGA that would be needed to obtain the results of alternative treatments because of its hepatotoxic effects (Lambert and Zhao, 2002).

Curcumin-treated MMT samples exhibited concentration-dependent inhibition of unregulated cellular growth. The drug's antiproliferative ability caused a significant inhibition of cell growth of more than 80% in the treated samples compared with the untreated control (Figure 3C).

The combination of tamoxifen and curcumin proved to be less effective than the use of tamoxifen or curcumin individually. The extent of inhibition of cell proliferation by the combination of tamoxifen and curcumin was less than the inhibition observed in cells treated with tamoxifen alone (Figure 4A). The tamoxifen and NDGA drug combination proved to be more effective. Dose–response showed significant inhibition in proliferation compared with the control population (Figure 4B).

ASSESSMENT WITHIN A RESEARCH SETTING

The breast cancer antiproliferative assay is one of several ongoing experiments that students complete during a Walter Reed internship. Students who participate in this investigation, as well as those who do not, complete additional studies in immunology, high-throughput cloning, neurotoxicity assays, physiological challenges, engineering problems, and so forth. These experiments, although varied, provide a unified experience for the students because each is built upon a fundamental concept, an accessible question, common protocols, data collection and graphing, mathematical analysis, and the formation of conclusions. Students become part of the training laboratory and do not receive class credit, grades, or content exams. Therefore, the assessment instruments target the internship experience as a whole, with an emphasis on the opportunity to observe,

think, extrapolate, validate, and skillfully conduct experiments. Furthermore, it is the students, not the senior researchers, who assess both the instructional experiments as well as the learning experience. Assessment thus becomes a process that is embedded in continuous input and culminates in a laboratory report or technical proficiency.

The following data are summarized from an external evaluation completed for the National Institutes of Health–Science and Education Partnership Award grant that funds this unique precollege/undergraduate research internship.

Student Assessment of the Research Experience

Precollege. Over the past 5 yr, 498 of the program's 735 students have come from a traditionally defined minority population, and female participation is consistently at 50% to 56%. These data, which mirror regional public school demographics, would indicate that gender and racial/ethnic inequality is minimal within the precollege program. Although the mammalian cancer assay was originally conceived to "grab attention" in light of the high breast cancer mortality rates within minority communities, our experience has demonstrated that scientific investigations consistently engage participants when targeted to any adolescent interest.

All students are treated like laboratory investigators, in that the more-focused students move more quickly to the next experiment and less-skilled students are given every opportunity to try again. As indicated in Figure 5, doing research science, regardless of the specific discipline, has measurable effects on student attitudes. The antiproliferative cancer assay requires a student to remain involved with the experiment for a week. This commitment to a single concept, combined with daily assessments of cell viability, allows students to assume responsibility for their own learning. Tools and techniques become the framework for critical thinking and the construction of explanations. Through peer-to-peer critiques, their skills are spotlighted and applauded.

As one part of an extensive pre-/post-2003 attitude survey, students were given seven questions that focused on whether they liked "doing" or "listening" or "watching." "Doing an experiment" with only a protocol was the overwhelming favorite, as seen in column three of Figure 5.

Although the above data demonstrate that the students obviously enjoyed a research internship, the question of their continued interest in the sciences can only be implied. Our program has expanded from six students to 216 students over 5 yr (waiting lists excluded). Currently, 100% of our senior students continue their education (20 seniors/yr only), 100% of returning students have taken another mathematics and/or science course, approximately 40% of our high school students participated in a science fair/building competition, and approximately 25% of the high school students returned for another year or more. Because of funding limitations, we can only anecdotally report that many students not returning to our program do attend a different summer internship. Furthermore, because the facility is not an accredited educational institution, legal barriers prohibit longer term data collection on minors.

Undergraduate. The breast cancer model has become a training tool for most undergraduates throughout the facility. During the external evaluation, 26 undergraduate researchers who had also participated in the precollege internships and the breast cancer investigation were asked to complete an eight-page postexperience survey.

These 26 undergraduate interns reported a high level of satisfaction with their internship experiences. Figure 6 provides a snapshot of their assessment. In the areas of skills acquisition, motivation, commitment, communications, leadership, and mentoring, the undergraduates felt that they had made significant progress toward their research and professional goals. These same 26 undergraduates were also mentors for the precollege students. In an effort to assess the undergraduate mentoring skills, the evaluator asked more than 200 precollege interns how they rated their undergraduate mentors. The final column of Figure 6 rates them as "awesome" (quote from a written response). The

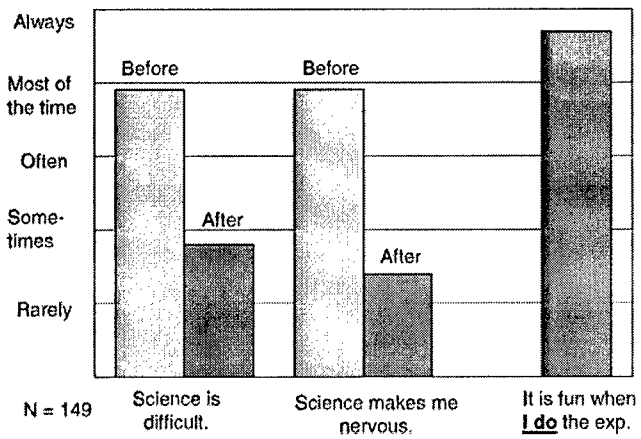


Figure 5. Student assessment of laboratory experiments, including the mammary cancer antiproliferation experiment. The 149 students took the same survey at the beginning and end of 1 wk of laboratory training. The survey used a Likert scale ranging from 1 (rarely) to 5 (always). This figure illustrates the effect that doing authentic experiments has on middle/secondary school students' attitudes toward science and research.

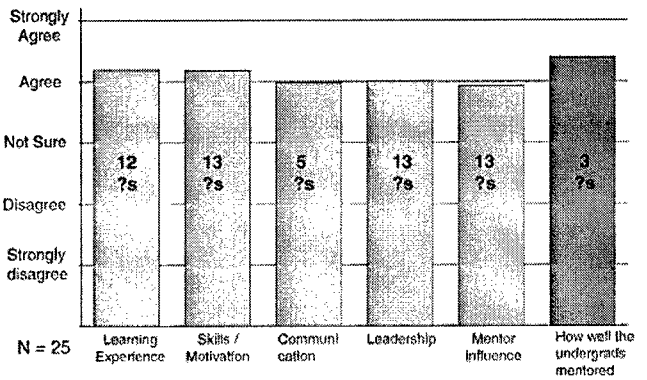


Figure 6. Undergraduate assessment of the effect of laboratory experiments used in training, including the breast cancer investigation. Various aspects of the internship experience were evaluated through clusters of questions. The numbers in the center of the bars represent the number of questions used to assess that particular cluster.

breast cancer investigation illustrates the dominant pedagogical methodology at the WRAIR: the more-experienced person mentors the less-experienced person in a continuous hierarchy on the basis of knowledge-supplementing skills and skills applied in a knowledgeable manner to the question.

A similar undergraduate survey was administered in a pre- and postformat to all undergraduate interns ($n = 82$) in 2003. A preliminary analysis indicates that Figure 6 reflects the undergraduates' feelings across the facility. Given current retention and dismissal rates within the facility, we estimate that 98% of the undergraduate interns are prepared for further responsibilities within a week. A facility-wide review showed that 38% of *all* our undergraduate interns originate in a traditional minority population. This suggests that many of our high school students advance to undergraduate work at the facility and that minority students regard the WRAIR as a known internship opportunity. In addition, 43% of our undergraduates return for an additional year or more of internship.

Formative Evaluation. At the precollege level, an anonymous 2004 evaluation showed that high school students, age 15–18 yr, generally found the technical difficulty of the breast cancer experiments to be appropriate. Students were also asked to rate their interest in the individual protocols on a Likert-type scale from 1 (not interesting) to 5 (very interesting). Cell culture and maintenance and the introduction to breast cancer research scored 4.3 ± 0.6, cell plating and the drug assay scored 4.1 ± 0.6, and analysis of the drugs and discussion of their nature scored 4.1 ± 0.5. Students often announce that plating cells is boring and drawing plots is hard. Yet many of these same students ask to return to test herbal remedies. They had experienced the process of cell biology and had become excited about discovery.

Every student who has ever participated in the antiproliferative drug assay has obtained results that could be displayed and then analyzed. The sense of accomplishment that a student gains from conducting "my own research" solidifies their learning in advanced topics such as apoptotic pathways or the genetic basis behind deregulated protein cascades. At the same time, the students' active involvement in protocol design and data gathering shifts the stress of learning from rote memorization to memorable initiative taking.

DISCUSSION

The rodent breast cancer cell experiment asks and answers one facet of a simple question: What have recent advances in genetics brought to the standard regimens used in treating breast cancers? In 5 d, in two 96-well plates, with readily available drugs, and at little more than the cost of media and cells, the student learns a range of laboratory skills and achieves competency in an adaptable yet common investigative assay. By focusing student attention on current pharmacological endeavors, the breast cancer study brings a relevant case study to the educational setting. The young researchers are excited, motivated, and frequently personally involved in the topic of cancer. Not only do students love the idea of working with an actual cancer model, they feel respected when given ownership of living cells and their fate.

The breast cancer experiment, as well as others designed by WRAIR scientists, follows a compromise approach between the "cookbook laboratory" and the "individually conceived science project." With our program, we learned early that "hands-on" is not enough to guarantee student learning. We noted that although students could be taught to manipulate equipment during an experiment, they could not necessarily apply their skills to a different project. These anecdotal observations, as reported by the scientists who helped launch the program, led to a change in the experimental emphasis from a hands-on to an inquiry method. As stated by both the American Association for the Advancement of Science (AAAS) and National Research Council (NRC) "inquiry is a set of interrelated processes by which scientists and students pose questions about the natural world and investigate phenomena; in doing so, students acquire knowledge and develop a rich understanding of concepts, principles, models and theories" (AAAS, 1993; NRC, 1995). The breast cancer experiment was one of the first investigations to diminish focus on technique to enhance the focus on concepts.

For the breast cancer cell training exercise, robust cells were selected to compensate for inexperience. Appendices became flexible so that more less-experienced students did less of the cell culture work and more-experienced students continued to maintain their own flasks. The structured daily protocols were refined, whereas the open-ended nature of the results was expanded and the initial emphasis on drug chemistry was replaced with the excitement of an unknown. These changes were quickly adopted by scientists at the WRAIR, who consistently work with students ranging from 16-yr-olds to fourth-year undergraduates. In fact, our investigators have revitalized their laboratory instruction by employing an inquiry emphasis on the breast cancer investigation and similar experiments (Shupp *et al.*, 2005). In the breast cancer study, their generalized knowledge base is placed in context and made relevant through the experience of personally witnessing the life and death of cells. Initially students are hesitant to begin an experiment that does not include an answer key. In point of fact, the older, more academically polished student usually experiences the greatest difficulty when handed the protocol. The instructor must be patient with the pleas of "What do you want me to do?" and "What if I mess up and do it wrong?" and an investigator's personal favorite, "What is the answer?" However, by the end of the second day, most of the students are completely engaged with their project and become offended if another intern/instructor intervenes without express permission.

The WRAIR, a premier research and training facility, has developed a student internship model and experiments that can be duplicated in any private or public research laboratory. From the research scientist's viewpoint, the learning objectives are straightforward: Can the intern assume independent responsibility for an experiment that integrates basic cell biology techniques with producing reproducible laboratory notes and a standard experimental summary? Using integrated, independent projects such as the breast cancer investigation, the senior scientist can assess the student's research acumen, initiative, and ability to work with the laboratory team.

ACKNOWLEDGMENTS

We thank the many mentors at WRAIR for their willingness to nurture a new generation of scientific researchers. We also thank the many undergraduate researchers who have taught this lesson to high school students in the GEMS program.

We thank the students and teachers at the Thomas Jefferson High School for Science and Technology in Fairfax, VA, and the McKinley Technical High School, District of Columbia Public Schools, for their support.

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Appendix A

MOUSE MAMMARY TUMOR CELL CULTURE AND MAINTENANCE

Mouse mammary tumor (MMT) cells are obtained from American Type Culture collection (ATCC, Manassas, VA). The cell Line Designation is: MMT 060562/Catalog No. CCL-51; mouse mammary gland tumor, epithelial morphology. Biosafety Level: 1.

Preparation of Media

The cells are grown in Eagle's Minimal Essential medium (EMEM) with Earle's BSS and 2 mM L-glutamine that is modified by ATCC to contain 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1.5 g/L sodium bicarbonate.

Before use, filter EMEM through a 0.2-μm filter unit and supplement with 50 mL of 10% fetal bovine serum and 5 mL of Pen-Strep (10,000 units/mL penicillin and 100 μg/mL streptomycin).

Handling Procedure for Frozen Cells

All steps should be carried out in a sterile biological hood. Upon receipt of the culture, the cells should be thawed rapidly and initiated to maintain the highest level of viability. If continued storage is necessary, the frozen culture should be stored in liquid nitrogen vapor phase.

1. Thaw the vial rapidly (within 2 min) by gentle agitation in a 37°C water bath.
2. Remove from the water bath and decontaminate the vial with 70% ethanol. All protocols should be followed under strict aseptic technique from this point on.
3. Prepare a 25-cm^2 tissue culture flask. Under strict aseptic technique, add approximately 5 ml of prepared medium. Because it is important to avoid excessive alkalinity of the medium during the cell recovery stage, place the culture flask containing the medium in the incubator for 15 min before adding the cells. This allows the medium to reach its normal pH of 7.0–7.6.
4. Transfer the vial contents to the 25-cm^2 tissue culture flask.
5. Incubate the culture at 37°C in a humidified 5% CO_2 atmosphere until a confluent monolayer is observed under a microscope (generally within 24 h).

Media Renewal

Media renewal should be done two to three times weekly. Media should be checked for:

- Clarity. The presence of debris or cloudiness is due to detached cells. This often occurs if the culture has grown so much that it can no longer maintain a confluent monolayer.
- Color. Cell medium is often supplemented with a pH indicator. Upon arrival, the bright red color of the medium indicates a neutral pH. Over the course of the week, the medium will progress from red to orange to yellow as a sign of increasing acidity.

Subculturing Procedure for Flask Cultures

Because these cells grow rapidly, it is recommended that subculture be done two times weekly at a ratio of 1:2. All volumes used in the following protocol are for a 75-cm^2 tissue culture flask and can be reduced or augmented as necessary.

1. Warm prepared cell media and trypsin in a 37°C water bath.
2. Wipe down the hood and all items inside with 70% ethanol.
3. If available, sterilize the bio-safety hood by turning on the ultraviolet light for 5–10 min.
4. Remove 25-cm^2 flasks containing the cell culture from the incubator.
5. Confirm cell confluence with an inverted microscope. Look for any possibility of contamination and evaluate the health of the cells.
6. Sterilize the outside of cell containers and your gloved hands with 70% ethanol, and place the flasks in the hood.
7. Use a pipette to remove the media from each flask and discard into waste beaker.
8. Pipette 15 mL of filtered phosphate-buffered saline (PBS) into each flask and wash the cells by holding the flask horizontally and slowly tipping it from side to side for about 30 s.
9. Remove PBS by pouring it into a waste beaker. Repeat steps 7 and 8 one more time.
10. Pipette 3 mL of trypsin-EDTA solution into each flask and expose the cells by holding the flask horizontally and slowly tipping it from side to side.
11. Let the flasks sit horizontally in the hood (~10 min) or back in the incubator (~5 min) until the trypsin begins to look cloudy.
12. Pipette 5 mL of medium into each flask to neutralize the trypsin.
13. Centrifuge at 3,000 rpm for 10 min. Resuspend the pellets in 10 mL of fresh sterile medium.
14. Incubate the cells in a 75-cm flask(s) in the incubator at 37°C in a humidified 5% CO_2 atmosphere.

Appendix B

PREPARATION OF STUDENT CELL CULTURE

On day 1, students receive their own aliquot of mouse mammary tumor (MMT) cells at a known concentration. Because the number of cells that goes into each well of a 96-well plate during the day 1 plating procedure must be constant, the concentration of the isolated cells must be obtained and modified before plating.

Cell Culture Detachment

Follow steps 1–11 of "Subculturing Procedure for Flask Cultures" in Appendix A for four flasks of MMT cells grown to a confluent monolayer.

Obtain two 50-mL tubes and divide the mixture of cells and trypsin-EDTA solution from each flask into the two tubes.

Balance the 50-mL tubes in a centrifuge and set the speed at 1,200–1,500 rpm and a temperature of 4°C for a period of 5 min.

Remove the tubes and look for a small white pellet of cells in the bottom. (If a pellet has not formed, continue to centrifuge the cells for an additional 5 min at the same settings.) Very gently pour off the supernatant, making certain the pellet stays in place.

Pipette 3 mL of medium into each tube and mix by gently pipetting. Combine suspended cells from both tubes into one tube.

Rinse the empty tube with 1 mL of medium; add this volume to the tube containing the suspended cells and vortex briefly.

Pipette 10 μL of suspended cells into a 1.5-mL tube. Add 90 μL of prepared trypan blue dye (0.4% trypan blue dye and sterile distilled water in 1:25 dilution). Vortex at a low speed.

The instructor calculates the cell concentration with a hemocytometer (see Appendix D, Day 3, for student review). Using the cell concentration worksheet (answer key below) ensure that there are 5×10^4 cells/mL for plating. If there are insufficient cells, the instructor should perform trypsinization on additional flasks or alter the number of cells that should be present in each well of the 96-well plate. The total amount of cell suspension required will depend upon the size of the class.

Cell Concentration Worksheet (Solution)

We want a concentration of 10,000 cells suspended in 200 μL of medium in each well of a 96-well plate. Find the concentration of cells per milliliter that is in each well. Show calculations below. (*Hint: 1 mL = 1,000 μL*)

$$(10{,}000 \text{ cells})/(0.2 \text{ mL}) = 5 \times 10^4 \text{ cells/mL}$$

$$\text{Desired cell concentration (cells/mL)} = \underline{5 \times 10^4 \text{ cells/mL}}$$

From the hemocytometer, a device used to measure the concentration of cells in a cell–medium suspension, the researcher obtains two cell counts.

$$\text{Cell count \#1} = 36 \text{ cells}$$
$$\text{Cell count \#2} = 30 \text{ cells}$$

Find the average cell count.

$$\text{Average cell count} = \underline{(36 + 30)/2 = 33 \text{ cells}}$$

The average cell count will allow us to calculate the concentration of cells in our cell–medium suspension. Earlier, we had done a 10:1 dilution with trypan blue dye, the dye that allows us to visualize cells on our hemocytometer. Therefore, we must now multiply the average cell count by 10.

$$(\text{Average cell count}) \times 10 = \underline{330}$$

Multiply this number by 10^4 cells/mL. This gives the concentration of cells in medium that we currently have.

$$\text{Current cell concentration} = \underline{330 \times 10^4 \text{ cells/mL}}$$

We have 6 mL of cell–medium suspension in our container. Multiply the concentration by 6 mL to obtain the total number of cells in the container.

$$\text{Total cells} = \underline{1.98 \times 10^7 \text{ cells}}$$

Remember from step 1 that we want to obtain a final cell concentration of $\underline{5 \times 10^4 \text{ cells/mL}}$ from our current cell concentration of $\underline{330 \times 10^4 \text{ cells/mL}}$. To do so, we must add medium to our 6 mL of cells to dilute to the desired concentration. Find the amount of medium we must add.

$$(\text{Total cells/desired concentration}) - (6 \text{ mL}) = \underline{390 \text{ mL of medium}}$$

However, we might not need nearly this amount of cell suspension. Say, for example, that 120 mL of cell suspension is all that is needed.

That could be obtained by taking 2 mL of cell suspension and adding 130 mL of medium.

Appendix C

DRUG STOCKS AND DILUTIONS

This appendix is designed for instructor or research assistant use only. Volumes can be augmented or reduced depending on the size of the class taught or the number of plates desired per student. Each weeklong experiment involves the use of the following five "unknown" drugs. The stock solutions for each drug are prepared according to the concentrations listed below and are thoroughly mixed before use to dissolve all solids. Tables 1–5 provide the concentrations to which the stock solutions will be diluted with EMEM medium. These concentrations can also be adjusted to provide more refined concentration curves. Refer to student handouts (Appendix D) for specific dilutions protocol.

Other drugs, such as 5-fluorouracil, can also be used effectively. Our facility has also tested simple pharmacological agents such as aspirin, hydrogen peroxide, antibiotics, and

Table 1. Curcumin stock solution: 10 mg/mL in 200-proof ethanol

Concentration (mM)	Medium	Stock solution
0.25	1,500	100
0.5	1,400	200
1	1,200	400
2	800	800

Table 2. Nordihydroguaiaretic acid (NDGA) stock solution: 15 mg/mL in 200-proof ethanol

Concentration (mM)	Medium	Stock solution
0.0125	1,599.6	0.4
0.025	1,599.2	0.8
0.05	1,598.4	1.6
0.1	1,596.8	3.2

Table 3. Tamoxifen stock solution: 250 mg/mL in dimethyl sulfoxide

Concentration (mM)	Medium	Stock solution
0.005	1,599.6	0.4
0.01	1,599.2	0.8
0.02	1,598.4	1.6
0.04	1,596.8	3.2

Table 4. Tamoxifen and curcumin stock solution: 18-μL tamoxifen stock solution (250 mg/mL in dimethyl sulfoxide) in 2.5-mL curcumin stock solution (10 mg/mL in 200-proof ethanol)

Medium	Stock solution
1,499.6	100.4
1,399.2	200.8
1,198.4	401.6
796.8	803.2

Table 5. Tamoxifen and NDGA stock solution: 18-μL tamoxifen stock solution in 18-μL NDGA stock solution

Medium	Stock solution
1,599.2	0.8
1,598.4	1.6
1,596.8	3.2
1,593.6	6.4

various herbs to give the students an opportunity to design "their own" study.

Appendix D
Student Guide

DAY 1

Introduction, Cell Concentration Calculations, and Plating Cells

Materials

Student handouts
Two 96-well plates/student or team
1 50-mL tube containing known concentration of MMT cells
1 15-mL tube containing sterile distilled water
Several repeaters
Bicomb Eppendorf tips for the repeater (1 = 100 μL)
Personal protective equipment (PPE): lab coat, goggles, gloves
Demonstration plate

Procedure

Background information on breast cancer and any applicable review.

Plating Cells: Students will plate their own cells. This protocol should be carried out under **sterile conditions** either on a clean bench or in a bio-safety hood. The students and instructor must wear gloves, lab coat, and goggles.

As an example for the class, the instructor should have a properly labeled demonstration plate on the bench.

Cell Plating

Take one 96-well plate into the sterilized hood. Open the packaging, being careful not to take the lid off of the plate.

1. Label the plates in small writing at the top with your names, the date, and name of the cell line. Also write initials on the side of the plate, while being careful not to jostle the lid on the plate. Draw a line over the very outer group of wells. These will be filled with sterile distilled water (evaporation of fluid from these outer wells makes the data from them undependable, so we simply add water to them).
2. Remove the lid and place it face up in the hood.
3. Place a tip into the Eppendorf repeater, being careful not to touch the tip and making sure that the tip locks into place.
4. Dispense 100 μL of distilled water per well to fill the outer wells.
5. Discard this tip into a sharps container.
6. Gently mix the cells by inverting the 50-mL tube several times. The aliquoted cell culture is provided by the instructor and labeled MMT.
7. Use a new tip to fill the inner 60 wells with 200 μL of cells in medium. On occasion, recap the tube and invert. If the cells are allowed to sit undisturbed, they tend to settle to the bottom, changing the cell concentration. Use a fresh tip when you are ready to continue pipetting.
8. When finished, discard the tip and place the lid back on the plate.
9. Repeat these steps for the second plate if necessary.
10. After all wells are filled, place them back into the incubator. The two plates can be stacked.
11. Clean your area with ethanol when finished.

Cells are incubated until ~70% confluence, usually 24 h (Figure 1).

DAY 2

Introduction to Drug Dilutions and Performing a Cellular Drug Resistance Assay

Materials

Drug Assay Worksheets
2 1.5-mL tubes each containing an unknown drug or combination of drugs
1 15-mL tube containing modified EMEM cell media
1 24-well plate
2 96-well plates containing confluent MMT cells (from day 1)
1,000, 100, and 10-μL pipettes with tips
Several repeaters
Bicomb Eppendorf tips for the repeater (1 = 50 μL)
PPE: lab coat, goggles, gloves
Demonstration plate

Procedure

Cell Concentration Worksheet. Students and teacher complete the formula sheet to calculate the final cell/medium suspension required for the sample number provided by the instructor. This can be done in the classroom or at the bench.

Drug Assay Worksheet. Each student will receive two drugs from the instructor for each 96-well plate. Fill out the appropriate Drug Assay Worksheet for each drug. These worksheets will be used later today.

The instructor should have a demonstration plate that illustrates how to label the additional information on the 96-well plate.

Each person has received two vials of unknown drugs. We will now prepare several different dilutions of each of the two drugs on 24-well plates. These will later be added to our plated cells.

Place the appropriate drug dilution sheets close by so they can be easily read.

1. Sterilize the workspace with 70% ethanol.
2. Carefully open the 24-well plate and label it according to the drug dilution sheet. As shown, to the far left, write drug number and medium type and then write the appropriate amount of the dilution and medium for that well.
3. Begin making the drug dilution by adding the appropriate amount of medium to the first well of the first drug dilution. This amount is specified on the Drug Assay Worksheet. Make sure to use the appropriate size of pipette and a sterile pipette tip.

4. Continue to add the appropriate amount of medium to the rest of the wells for the first drug's dilution. Dispose of the tip in the sharps bin.
5. Next, add the appropriate amount of the drug into the first and second wells of the plate. Dispose of this tip.
6. Mix the dilution in the first well by pipetting up and down several times with a new tip. Add the appropriate amount of this dilution to the third well. Dispose of this tip.
7. Take another new tip and mix the dilution in the second well by pipetting the solution up and down several times. Add the appropriate amount of this dilution to the fourth well.

This procedure can be repeated to make the second drug's dilution.

Performing the Drug Assay or Plating the Dilutions

1. The instructor will demonstrate this procedure for adding the drug dilutions to the 96-well plates, before the students do the same to their own plates. The Drug Assay Worksheet should be used as a reference. After the instructor is done, he or she will come around and check the students' Drug Assay Worksheets to make sure that they are properly filled in. Then the instructor will remove the students' plates from the incubator and distribute them so the students can begin the procedure. Remove the 96-well plates from the incubator and place on a sterilized surface.
2. There will be 10 columns of cells with four wells per column. For beginning students, use two columns for each dilution of the drug (eight replicates). For more experienced students, one column or four replicates is usually sufficient. Label each plate with the appropriate drug number (the number on your vial) and drug dilution number as shown on the Drug Assay Worksheet. A diagram of a labeled 96-well plate is shown on the worksheet.
3. Place a fresh sterile tip into a repeater. Set the dial so that 50 μL will be dispensed with each click.
4. Take one plate aside and remove the lid, placing it face up in the clean/sterile work area.
5. Add 50 μL of the first drug dilution to the first two columns of the cells.
6. Change the repeater tip and add 50 μL of the second dilution to the third and fourth column of the cells.
7. Continue changing tips for each dilution and continue adding each dilution to the appropriate columns of cells.
8. There were four dilutions of each drug and five pairs of columns per drug. The fifth pair of columns is for medium to be added to the cells with no drug. This is the control set of wells.
9. Place the lid on the plate and place this plate aside in the incubator.
10. Repeat the procedure if necessary to the second 96-well plate.

When finished, place all plates back into the incubator. Clean up the area and wipe all surfaces down with 70% ethanol.

DAY 3

Your class will receive an aliquot of MMT cells of unknown cell concentration. Using a hemocytometer, you will calculate the cell concentration as well as the dilution required to achieve the required 10,000 cells suspended in 200 μL of medium. Once the cells are diluted and concentration is confirmed, the cells can be returned to a tissue culture flask.

Using a Hemocytometer to Estimate Cell Concentration

Pipette 10 μL of suspended cells into a 1.5-mL tube. Add 90 μL of prepared trypan blue dye (0.4% trypan blue dye and sterile distilled water in a 1:25 dilution). Vortex at a low speed.

Place a coverslip over the slide with the hemocytometer grid. Pipette 10 μL of the cell/dye dilution into each side of the slide.

Focus the microscope on the hemocytometer grid and bring the upper left square of the large grid square into view.

Begin to count the cells found in this section of the hemocytometer grid. Record this number and move the slide to count the number of cells in the lower left corner, upper right corner, and lower right corner, recording the additional values as you continue.

Add the recorded values together and divide by four to get an average number of cells per square. This value will be known as **cell count 1.**

Move the focus of the microscope to the second grid of the hemocytometer and repeat this method of counting to find a second average number of cells per square for the upper half of the instrument. This value will be known as **cell count 2.**

Cell Concentration Worksheet

We want a concentration of 10,000 cells suspended in 200 μL of medium in each well of a 96-well plate. Find the concentration of cells per milliliter that is in each well. Show calculations below. (*Hint: 1 mL = 1,000 μL*)

Desired cell concentration (cells/mL) = _____

From the hemocytometer, a device used to measure the concentration of cells in a cell–medium suspension, the researcher obtains two cell counts.

Cell count #1 = 36 cells
Cell count #2 = 30 cells

Find the average cell count.

Average cell count = _____

The average cell count will allow us to calculate the concentration of cells in our cell–medium suspension. Earlier, we had done a 10:1 dilution with trypan blue dye, the dye that allows us to visualize cells on our hemocytometer. Therefore, we must now multiply the average cell count by 10.

(Average cell count) × 10 = _____

Multiply this number by 10^4 cells/mL. This gives the concentration of cells in medium that we currently have.

Current cell concentration = _____

We have 6 mL of cell–medium suspension in our container. Multiply the concentration by 6 mL to obtain the total number of cells in the container.

Total cells = _____

Remember from step 1 that we want to obtain a final cell concentration of _____ from our current cell concentration of _____. To do so, we must add medium to our 6 mL of cells to dilute to the desired concentration. Find the amount of medium we must add.

(Total cells/desired concentration) – (6 mL)
= _____

DAY 4

Using data from the previous year or demonstration plates, you will learn how to read fluorescent scan spreadsheets and how to do a simple analysis. The breast cancer model has traditionally used CyQUANT because of its simplicity, but other proliferation assay kits would be just as effective. Your instructor will review the product literature to help you determine what is being measured. Your instructor will demonstrate how to use the scanner or plate reader to acquire and analyze the image. Also, the instructor will explain how to save the data in a tab-delimited format and use spreadsheet data analysis software (MS Excel, SigmaPlot) to plot the data and carry out some statistical analyses, such as averaging the data. Cell cultures may be frozen at this point.

DAY 5

Cell Proliferation Assay, Scanning, and Analysis

Materials

CyQuant Cell Proliferation Assay Kit (consists of components A and B)
2 96-well plates incubated with drug assay
1,000-μL pipette with tips
Eppendorf repeater with 100-μL tips
1 50-mL tube wrapped in aluminum foil
Sterile distilled water
Scanner, inverted microscope

Procedure

Part 1: Preparation for Scanning. Prepare the plates for scanning with the CyQuant Cell Proliferation Assay Kit.

1. Remove both 96-well plates from the incubator. Remove the lids and invert both plates onto an absorbent pad to remove all of the medium. Shake vigorously to ensure complete medium removal.
2. Stack plates right side up and place in the freezer for at least 30 min (overnight is fine).
3. Obtain a 50-mL tube and wrap in aluminum foil. This tube will contain the dye mixture.
4. Mix 2 mL of buffer stock (component B) with 38 mL of distilled water in the 50-mL tube.
5. Add 100 μL of CyQuant GR stock solution (component A) to the tube. Vortex.
6. Using a repeater, add 200 μL of your dye mixture to each sample well.
7. Incubate for 5–20 min at room temperature and minimal light exposure.
8. The plates are now ready to be scanned using the BioRad software. They will be read at 480 nm excitation and 520 nm emission. A low fluorescence reading specifies cell death, indicating a potential drug for treatment, whereas higher fluorescence specifies viable cells, indicating an ineffective drug or drug dosage against these cells.

Part 2: Analysis. Students learn how to export their data from the scanner and how to use a spreadsheet, analyze the data, and create plots. Each drug is added at increasing concentrations in four to eight wells for each concentration. Students calculate the average cell number for each concentration and divide it by the cell number in the control wells. This yields the percentage of viable cells for each treatment. The results are plotted and compared with standard curves that have been previously established by the instructor. Once the students have matched their plots to the standard, the names and nature of the drugs are revealed and discussed. The students then discuss effective drug or drug combination(s) on the basis of cell proliferation.

Appendix E

Student drug assay worksheet: The worksheet serves as a student protocol as well as a teaching instrument. Students calculate the volume of drug stock needed to complete the table. The sheet varies for each drug. In this example, the student is given curcumin and NDGA.

Drug Assay Worksheet

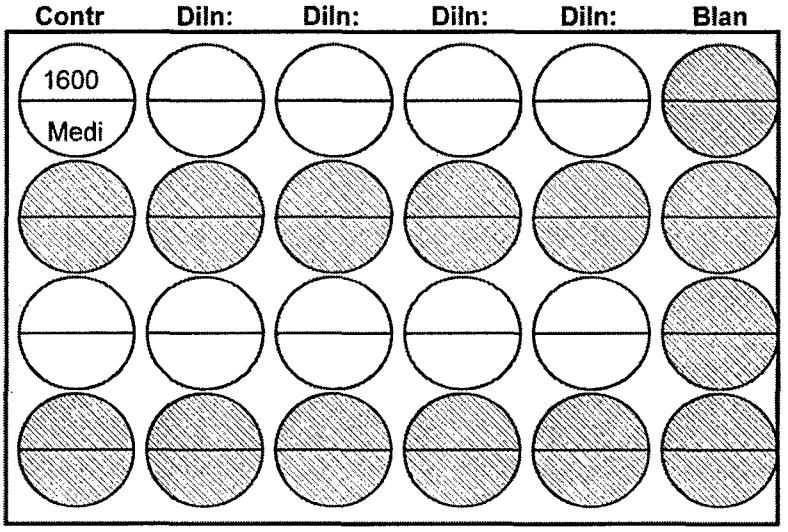
Name _____

You have been given two small vials of unknown drugs #1 and #2. We want to dilute the drug stock with media so that there is a total of 1600 uL in each well of the 24-well plate. With this information, complete the charts below.

Drug	Final conc [mM]	Dilution	Media (uL)	Drug Stock
#1	0	C	1600	
	0.25	1	1500	
	0.50	2	1400	
	1.00	3	1200	
	2.00	4	800	

Drug	Final conc [mM]	Dilution	Media (uL)	Drug Stock
#2	0	C	1600	
	0.0125	1	1599.6	
	0.0250	2	1599.2	
	0.0500	3	1598.4	
	0.1000	4	1596.8	

Add the calculated amount of drug stock and the corresponding amount of media to each well of a 24-well plate according to the diagram shown below. Pipette up and down to ensure even mixing. The grayed-out wells should be left blank.



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Expression patterns of Fatty acid binding proteins in breast cancer cells

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We studied the expression levels of fatty acid binding proteins in breast normal and cancer cell lines. Liver fatty acid binding protein (L-FABP) and intestine fatty acid binding proteins were shown to be up-regulated in breast cancer cell lines while adipose- and epidermal-fatty acid binding proteins were down regulated in breast cancer cells compared to normal breast cell lines. We have previously shown that blocking the expression of L-FABP resulted in remarkable effects on apoptosis and cell proliferation of prostate cancer cell lines (Hammamieh et al 2004). To study the mechanism of effect of the liver fatty acid binding protein in breast cancer cells, we designed an antisense oligodeoxynucleotide to block the production of liver-FABP in MCF-7 cells. The antisense was shown to inhibit the expression of L-FABP and to induce apoptosis in MCF-7 cells.

This study examines the mechanism by which L-FABP antisense regulates proliferation and apoptosis in breast cancer cell lines. We used human cDNA microarrays to explore differentially expressed genes in MCF-7 breast cancer cells treated with L-FABP antisense oligonucleotide. Some of the genes that were differentially expressed were confirmed using quantitative RT-PCR. Genes related to cell growth, proliferation and angiogenesis showed significant variations. This suggests a possible use of these antisense ODNs as therapeutic agents for breast cancer in future.

Key words: breast cancer, fatty acid binding proteins, L-FABP, apoptosis, microarray, MCF-7

INTRODUCTION

Fatty acid binding proteins have long been studied for their role in solubilization and transport of fatty acids from the cell membrane to different cellular organelles (1). Although FABPs have been described as cytosolic proteins with low molecular weights (around14 kDa) encoding about 115 amino acids, it has been suggested that these proteins are possibly major components of membrane proteins (2).

Prior data reported a well-established relation between certain FABP levels and oncology, though the mechanism is still poorly characterized. For example: the expression of the Heart-FABP, also known as mammary-derived growth inhibitor (MDGI) is absent in tumor cells and is highly expressed in normal lactating mammary cells (3). Elevated expression levels of L-FABP were detected in liver cell lines during cell proliferation such as carcinogenesis or regeneration (4).

Previous studies in our laboratory showed that the expressions of certain FABPs were altered in prostate cancer compared to normal cells. Our studies revealed a delicate balance between the levels of good FABPs vs. the bad FABPs in normal and cancer cells. A-FABP, which is closely related to H-FABP and E-FABP were down regulated in prostate cancer cells, whereas the levels of L-FABP and I-FABP were up regulated in these cells (5, 6). When prostate cancer cells were treated with L-FABP antisense oligodeoxynucleotide in order to diminish the expression of L-FABP, it exhibited an enhanced apoptosis and inhibition of proliferation in tumor cells (7).

Table1. *The list of the primers used in this study.*

PRIMER NAME	5'-FORWARD PRIMER-3'	5'-REVERSE PRIMER-3'
FPR1	TTCCGGATGACACACACAGT	TCCGAACAAGTTGATGTCCA
PFN1	GGGAAAACGTTCGTCAACAT	ACACCTTCTTTGCCCATCAG
TTF1	CACTACCACACCCGGCTAAT	GCAAGTTTTCCCATTTCGAG
ERG-1	GGAGTTTCTTCCGCATGTGT	AGTCTCGCTGCAATCAAGGT
THBS1	TGATAGCATAGGGGAGGCC	TCCTGTGATGAGCTGTCCAG
SEPN1	CTTCTAAAACTTGAGTGGCTGTC	CTTCTAAAACTTGAGTGGCTGTC
K-ALPHA-1	TTATCCATTCCTTTTGGCCCT	CATGGAAAAGACATGATCACCAA
MAPDD	GGGAGTTGAGGACATTTATTTCA	ATCAGAGGATGGAGTGGTCG
HDAC1	TATCTCAAAAAGGAAACTAGACT	CTGCTTAGTAGCTTTGGA
TERT	TACGTCGTGGGAGCCAGAAC	CCTTCACCCTCGAGGTGAGA
CKMT	GGCACAATGACAATAAGAC	TTCCACATGAACTCATAGT
YWHA1	TTCAATGCAATGCATCTG	CGCATTTATTTACCCATTGA
SP2	GAAAACCGCTGGCAAGGAAAC	CCTGTACCCACCTCCATC
IFN-A	TCCTAAAAATACGGGCAAGC	AATTCCCAGACTTCCTGCCT
IFN-G	CTGCCTCTAAAAGGCCTGTCC	GACCAGGTCTTCTGACTGCTCA
IL-6	GGTGGCAAAAAGGAGTCACA	TAGAATTTAGCGTTCCAGTT
TNF-B	TCTCGAACCCCGAGTGACAA	TATCTCTCAGCTCCACGCCA
TGFB2	TCTTGGAAAAGTGGCAAGAC	CCAAAGTGTCTGAACTAGTACCG
IGFR	CTTTCCAGAAACTCTGTGTAAAT	CTGTTTCTCACAGAGTATTTTCA
EGFR	TATTTTTGTGGCCAAATCAGG	GCTCCAACCTATCACCTAGCA
FGF	ATAAAGCAGCTTTTCATGAATGC	ACTTTGGATTTTGCAATGCC
18S	AATTGACGGAAGGGCACCAC	CGGACATCTAAGGGCATCACAG
A-FABP	GAAACTTGTCTCCAGTGAAAAC	GGGAGAAAATTACTTGCTTG
E-FABP	CCGACGCAGACCCCTCTC	GATCCGAGTACAGGTGACATTGTTC
L-FABP	CTCTATTGCCACCATGAGTTTC	GCTGATTCTCTTGAAGACAATCTG
I-FABP	GCGCACAGTCCAAAATACAA	TTAGCTTTTACTTCTTTTGCTTTGG

In the present study, we have examined the expression levels of FABPs in MCF-7 and T-47D breast cancer cells compared to normal breast cells. Although the different FABPs share a low to moderate sequence homology (20-70%), the present data suggests that they exhibit a highly variant expression pattern in tumor cells. Adipose- and epidermal-FABP were down regulated in MCF-7 and T-47D breast cancer cells whereas L-FABP and I-FABP were up regulated in these cells compared to normal breast cells.

In order to understand the mechanism of FABP action we have used antisense oligodeoxynucleotides (ODN) to L-FABP in MCF-7 breast cancer cells to lower the levels of expression and studied the molecular changes in gene expression of L-FABP patterns and induction of apoptosis. The results clearly suggest that L-FABP plays a role in cell proliferation and apoptosis in breast cancer cells.

MATERIALS AND METHODS

Tissue culture media, custom oligonucleotide DNA primers, TRIZOL and reverse transcriptase were obtained from Invitrogen, Inc. (Gaithersburg, MD). PCR Master Mix was obtained from Boehringer Mannheim (Indianapolis, IN).

Cell and Tissue Samples

Culture conditions and media used for individual cell lines were prepared as described in the manufacturer's instructions (American Type Culture Collection, Manassas, VA, and Cambrex, respectively) and each cancer cell line was cultured in the appropriate culture fluid containing 5% fetal bovine serum. Primary cultures of normal mammary epithelial

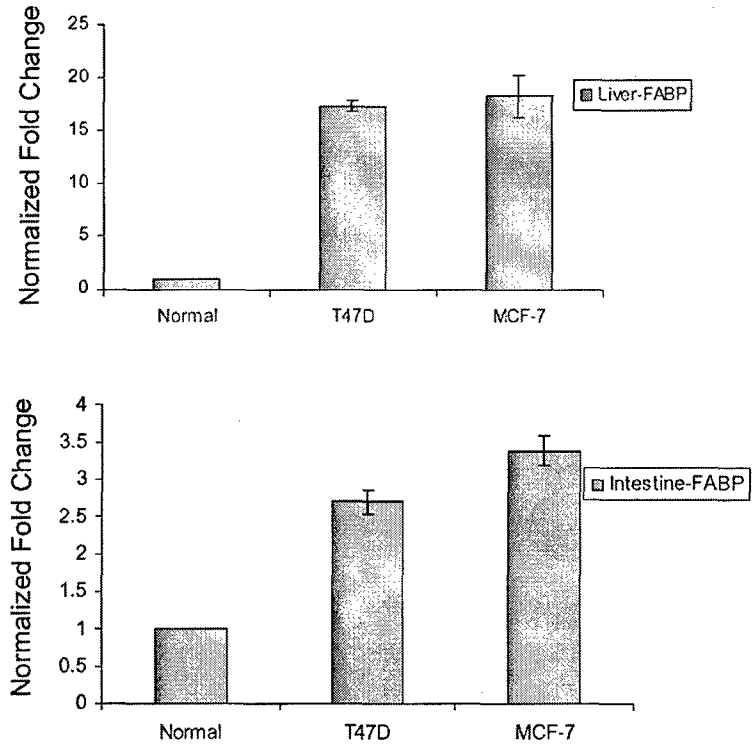

Figure 1. Expression levels of liver-FABP (a) and Intestin-FABP (b) in cultures of normal and cancerous breast cell lines. Breast cells were cultured, and total RNA was isolated using the Trizol method. Equal amounts were taken to determine gene expression levels using the specific primers (Table 1) and performing RT-PCR. The PCR product was resolved on a 1% agarose gel, and intensities were measured using QuantityOne software (BioRad).

cells (HMEC) were obtained from Cambrex and cultured in the fluid formulated especially for these primary cell cultures.

RT-PCR Analysis of FABP mRNA

Total RNA samples were isolated from cells using the TRIZOL method according to the manufacturer's instructions (Invitrogen, Inc.) with some modifications. The RNA samples were treated with DNase-1, to remove genomic DNA, and re-precipitated. Reverse transcription and subsequent polymerized chain reaction (PCR) of FABPs were performed using specific primers for each transcript (see Table 1). The homology of PCR products with the corresponding GenBank sequence for each FABP was verified by sequencing the PCR products using a Cycle sequencing kit (Amersham, Arlington Heights, IL).

Quantitation of PCR Products

Different numbers of PCR cycles were used to determine the cycle number at the linear range of PCR products. That was determined to be 30 cycles for the FABP genes and 18 cycles for the 18S housekeeping gene. Equal amounts of RNA samples were used to perform the RT-PCR reaction for all of the genes. Samples were resolved on agarose gels, scanned, and digitized using a Multifluor FX scanner and Quantity One program (BioRad, Hercules, CA). For each RNA sample, a parallel reaction containing primers for 18S ribosomal RNA was performed as an internal control for normalization of samples.

TREATMENT WITH ANTISENSE OLIGONUCLEOTIDE

For antisense studies, phosphorothioate oligonucleotides were commercially synthesized (KamTek Inc., MD). MCF-7 breast cancer cells were plated and 10 μM of the oligodeoxynucleotides was added. The sequences of the antisense and sense oligos are as follows: antisense for L-FABP is 5'-CTACAGGAATACGCA-3', sense for L-FABP is 5'-TGCGTATTCCTGTAG-3'.

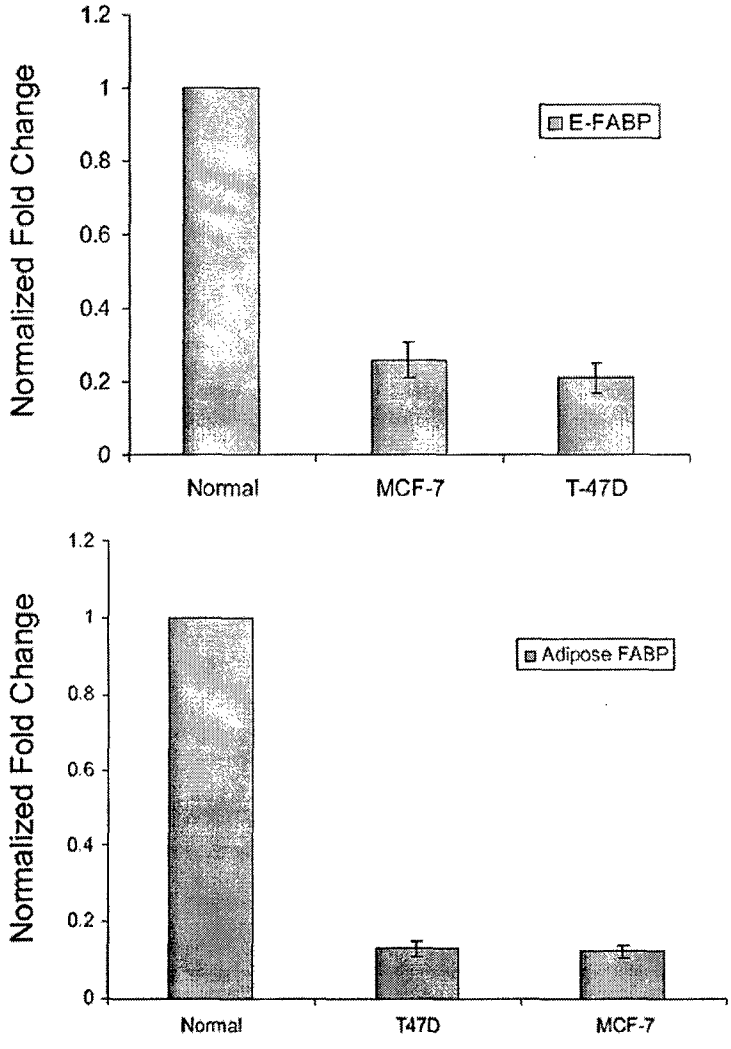

Figure 2. Expression levels of epidermal-FABP (a) and adipose-FABP (b) in cultures of normal and cancerous breast cell lines. Breast cells were cultured, and total RNA was isolated using the Trizol method. Equal amounts were taken to determine gene expression levels using the specific primers and performing RT-PCR. The PCR product was resolved on a 1% agarose gel, and intensities were measured using QuantityOne software (BioRad).

Apoptosis Study

MCF-7 cells were plated overnight on an eight-well chamber slide. The next day, 10 μM L-FABP antisense was added, and cells were incubated for 48 h. The cells were then fixed with paraformaldehyde and stained with Hoechst 33258 dye for 30 min. Cells having bright, fragmented and condensed nuclei were identified as apoptotic cells. The number of apoptotic cells was counted in 10 microscopic fields (x40) in each case.

Microarray

Total RNA was isolated separately from cultured MCF-7 cell line and the same cell treated with 10 uM antisense ODN of L-FABP following TRIZOL technique. The quality and quantity of isolated RNA was evaluated by Bioanalyzer 2100 (Agilent, CA). A custom made cDNA library containing 7489 genes, including 7019 known genes, 249 unknown genes and 110 ESTs, was used for the microarray experiment. Samples were labeled with Cy3 dye using the TSA labeling kit (Perkin Elmer, Boston, MA) and co- hybridized with a reference RNA (Strategene, CA) labeled. Each experiment was

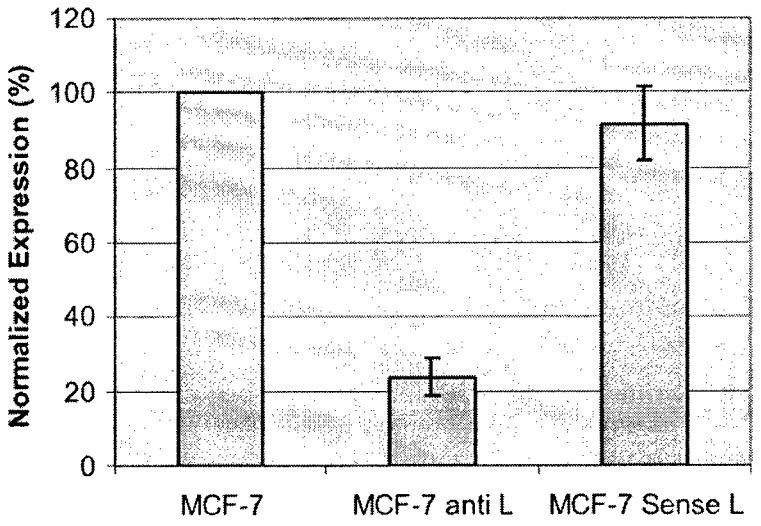

Figure 3. Effects of the antisense and sense ODNs on the expression levels of L-FABP in MCF-7. MCF-7 cells were incubated +/- 10 μM of each of the oligos for 48 hours. Total RNA isolated using the Trizol method. Equal amounts were taken to determine gene expression levels using primers specific for L-FABP and for performing real-time RT-PCR.

carried out in duplicate. The array images were scanned by GENEPIX PRO 4100b (Axon Instruments, Inc., CA) optical scanner and the image analysis were performed using GENEPIX PRO version 4.

Data filtering and analysis

Data from microarray experiments were preprocessed and analyzed by using GeneSpring (Silicon Genetics, CA) version 7.0. The raw intensity, recorded for each color channel, was filtered individually to select the entries featuring intensity at least 2 fold higher than the corresponding background level. Intra-chip LOWESS normalization was carried out over the noise-free genome set and the normalized data was expressed as the $\log_2$ ratio of normalized intensity of treated sample against that of control sense ODN treated sample. The genes showing significant variability ($P < 0.05$) from the baseline were selected by analyzing the variance of genomic regulation (ANOVA) between control and treated samples.

Real time PCR

Real time polymerase chain reaction was carried out in BIORAD I-cycler using SYBR green I kit (Roche Diagnostics, Indianapolis, Indiana) following the manufacturer's recommendation. The 18 S primers were used as control to normalize the raw real-time PCR data. Five technical replicates were carried out for each primer set. The threshold cycle (Ct) for every run was recorded and was duly converted to the fold change through the following equation: $[(1 + E)^{\Delta Ct}]_{GOI} / [(1 + E)^{\Delta Ct}]_{HKG}$; where ΔCt denoted the difference between the Ct of control and treated samples of a given gene and GOI and HKG defined as House Keeping Genes and Genes Of Interest respectively. E symbolized the primer efficiency, calculated from the slope of the best fitting standard curve of each primer.

RESULTS

Expression patterns of FABPs in breast cancer cells

We studied the expression levels of four FABPs, adipose fatty acid binding protein (A-FABP), epidermal fatty acid binding protein (E-FABP), intestine fatty acid binding protein (I-FABP), and liver fatty acid binding protein (L-FABP) in MCF-7 and T47-D cell lines compared to the normal HMEC epithelial cells.

Polymerized chain reaction revealed that the expression of both L-FABP and I-FABP were up regulated in MCF-7 and T47-D when compared to normal breast cells (Fig. 1a, b). However, a significant down regulation in the expression levels of A-FABP and E-FABP was observed (Figure 2a, b). Similar patterns of FABP expression were observed in prostate cancer cells when compared to normal epithelial prostate cells in vitro and in human biopsy samples (Das 2001).

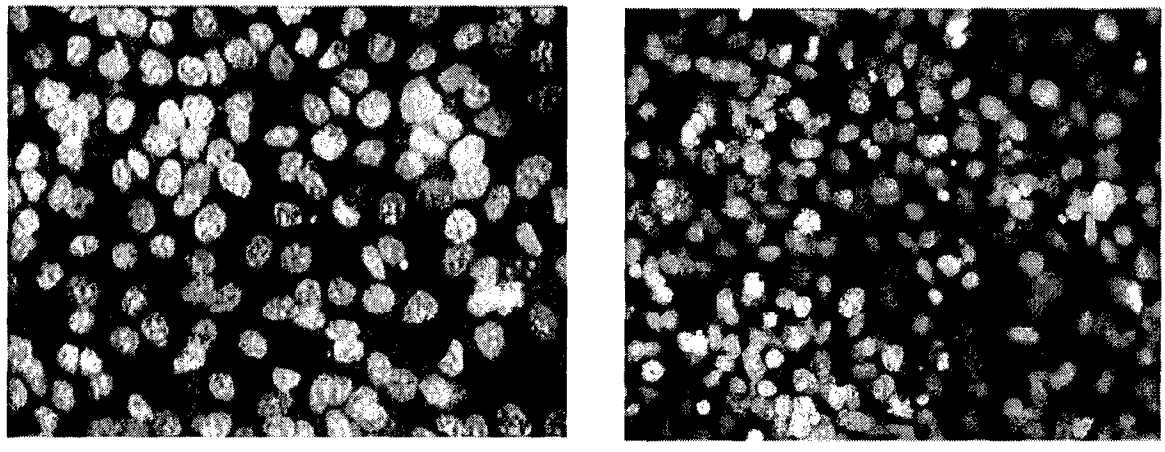

Figure 4. Effect of antisense ODN constructs of L-FABP on cell apoptosis. MCF-7 cells were treated with antisense ODN to L-FABP at 10 μM concentration and incubated for 48 hrs. Cells were fixed and stained with Hoechst to detect apoptotic cells.

Study of the effect of L-FABP antisense on the expression of L-FABP in MCF-7 cells

MCF-7 cells were exposed to antisense ODN to L-FABP and its sense ODN for 48 h. RT-PCR was carried out using the primers specific for L-FABP. The expression levels of L-FABP showed more than 70% decrease in MCF-7 cells treated with L-FABP antisense compared to sense L-FABP–treated cells (Figure 3).

Induction of apoptosis by L-FABP antisense ODN

MCF-7 cells were stained with the DNA binding dye Hoechst to determine the number of apoptotic cells. Non apoptotic cells displayed intact regularly shaped nuclei and normal chromatin distribution under fluorescent microscope; while apoptotic cells with compacted and segregated masses of chromatin and nuclei, displayed a non regular diffused shape. When MCF-7 cells were exposed to the L-FABP antisense ODN, the percentage of apoptotic cells was increased by more than 10 fold with respect to control cells. However, there was no noteworthy increase in apoptotic features when cells were treated with the sense ODN (Figure 4).

Effect of L-FABP antisense ODN on gene expression patterns in MCF-7 cells

We used cDNA microarray to understand the mechanism of effect of the L-FABP antisense in MCF-7 breast cancer cells. RNA samples were isolated from both treated and untreated cells, while the former cells were subjected to 48 hours incubation with 10 μM antisense ODN to L-FABP prior to the extraction. The RNA was, subsequently, qualitatively and quantitatively validated, labeled and eventually hybridized on cDNA microarray. Data were analyzed using GeneSpring 7.0 (Silicon Genetics, CA). Analysis of variance was applied to identify the statistically significant genes that were altered with a p-value <0.05 within the control and treated samples. Data obtained from microarray experiments were confirmed for expression levels of selected genes using real time PCR.Genes that were up regulated by L-FABP antisense treatment in MCF-7 cells include estrogen regulated gene 1 (ERG1, ID: AI417638), interferon induced transmembrane protein (IFITM1, ID: AA862371), guanylate cyclase activator 1A (GUCA1A, ID: AA431439), ATPase Ca++ transporting protein(ATP2A3, ID: AI038202), prostate cancer tumor suppressor protein (TUSC3,ID:N66008), transcription termination factor (TTF1, ID:AA709143), inhibin beta B (INHBB, ID: AA431428), Profilin1 (PFN1, ID:AA521431), Interferon alpha(IFN-a, ID:S78750), interferon gamma (IFN-g, ID:AA969504), interleukin-6 (IL-6, ID:N98591), inducible nitric oxide synthase (NOS2A, ID:AA877840) and tumor necrosis factor beta (TNF-b, ID:S45212).

We used PCR and real time PCR to confirm the observed up regulation of a few selected genes. Figure 5 shows the expression profiles of IFN-a, IFN-g, IL-6, INOS and TNF-b confirmed using PCR.

We have carried out real-time PCR to confirm the up regulation of estrogen regulated gene 1, formyl

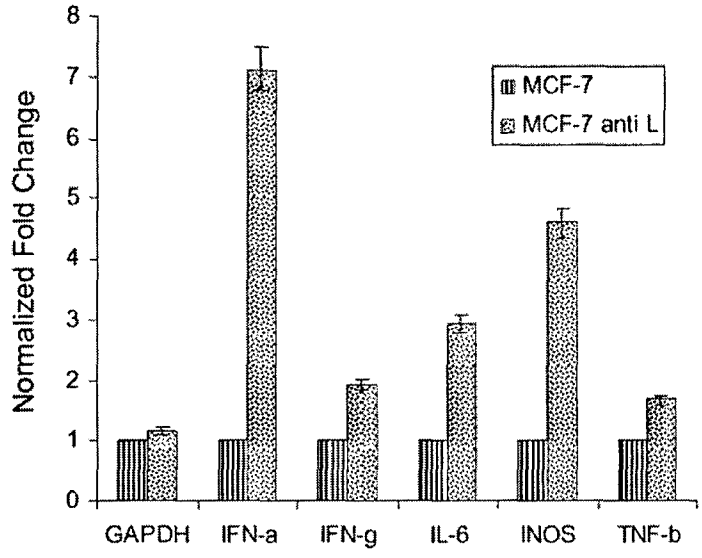

Figure 5. Confirmation of the expression patterns of genes identified by cDNA microarray. MCF-7 cells were cultured and total RNA isolated using the Trizol method. Equal amounts were taken to determine gene expression levels using primers specific for each gene and performing RT-PCR. The PCR products were resolved on a 1% agarose gel and intensities were measured using QuantityOne software (BioRad) and normalized to actin and/or S18 ribosomal protein.

peptide receptor 1, profilin 1 and transcription termination factor. The real-time PCR results for these up regulated genes are expressed in figure 6. Some of the down regulated genes include solute carrier family 6 (SLC6A6, ID: AI949407), zinc finger protein 212 (ZNF212, ID: AA457155),MAP-kinase activating death domain (MAPDD, ID: AA281945), carbonic anhydrase XI (CA11, ID: N52089), Sp2 transcription factor (SP2, ID: T81103), histone deacetylase 1(HDAC1, ID: AA465353), selenoprotein 1 (SEPN1, ID:AA070226), creatine kinase (CKMT, ID: AA019332), heat shock 10kD protein 1(HSPE1, ID: AA448396), telomerase reverse transcriptase (TERT, ID: AI824948), tyrosine 3-monooxygenase (ID:AA 63399), cetromeric protein F (ID: AA701455), alpha-tubulin (k-alpha-1, ID:XM_084866), cyclin B2 (CCNB2, ID: AA774665). Their gene expression levels are illustrated in figure 7.

Furthermore, a group of growth factors were found to be differentially expressed in MCF-7 treated with the L-FABP antisense and sense ODNs (Figure 8).

DISCUSSION

Liver fatty acid binding protein (L-FABP) belongs to a protein family, collectively known for transporting of bioactive lipids within cells. By keeping an association with 60% of cytosolic long chain fatty acids, L-FABP performs an important storage function by maintaining an intracellular pool of essential fatty acids (8).

The role of Liver-FABP in carcinoma cell was under scrutiny for a long time. Although the mechanism is still poorly understood, its relation with tumorognesis is evident. Previous studies have described L-FABP to stimulate cell growth when transfected into hepatoma cells (9, 10). L-FABP was reported as highly expressed in intestinal metaplasia and in a subset of gastric adenocarcinomas (11). We have previously studied the expression pattern of L-FABP in prostate cancer and shown that the levels of L-FABP were highly expressed in cancer cells and tumor biopsy samples compared to normal cells (5).

We have used an antisense ODN to inhibit the expression of L-FABP in prostate cancer cells and showed that the antisense inhibited proliferation and induced apoptosis in these cells (7).

In this study we have shown that fatty acid binding proteins are also expressed at varying levels in breast normal and cancer cell lines. L-FABP was again found up regulated in MCF-7 breast cancer cells, which led us to use a pre-validated antisense to L-FABP (7) to block its expression in tumor cells. Thus we expected to induce apoptosis in these cells. The cell morphological pictures (Fig 4) depicting the cells stained with the Hoechst DNA binding dye confirmed that inference. In order to further investigate, on the molecular levels, L-FABP's role in breast cancer, we

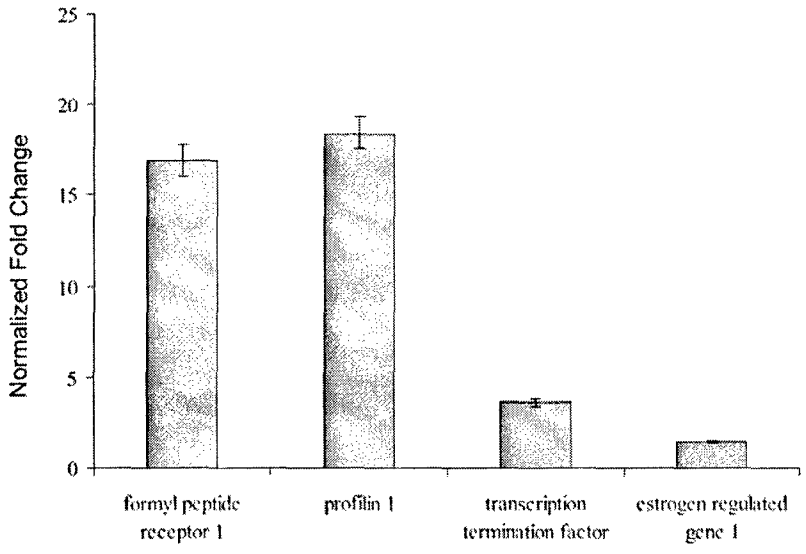

Figure 6. Study of the expression levels of genes up regulated by the antisense using real time PCR. Some of the genes that were shown to have altered expression levels using cDNA microarray were confirmed using real time PCR. Data represented here are the expression levels after normalizing to actin and S18 genes.

used cDNA microarrays to study gene regulations in MCF-7 cells exposed to the antisense ODN. The rationale for the use of global gene analysis was to understand how this one protein can alter cancer cells and what other pathways does this protein target in breast cancer cells. The genes found to be regulated by the antisense include a number of apoptosis and cell-growth regulator genes. Three members of the interferon family, namely alpha, gamma interferon and interferon induced transmembrane protein 2 were up regulated in the presence of L-FABP antisense. They are essential for induction of apoptosis through arresting the tumor development and mediating the cell-proliferation. Interleukin-6 and tumor necrosis factor beta showed similar regulation trend. The former suppresses proliferation and metastasis in cancer cells (12, 13) and the latter sets off apoptosis through extrinsic or death receptor pathway (14). BAX inhibitor 1 (BI1; ID: AA629591), known for suppressing intrinsic or mitochondrial apoptosis triggered by BAX (15), was down-regulated in the presence of the antisense. One more down-regulated gene, CD28 antigen (Tp44; ID: AI375736) has been identified as instrumental factor to delay the onset of cellular apoptosis (16). Suppression of another anti-apoptotic gene, MAP kinase-activating death domain further endorses L-FABP antisense's anti tumorogenic characteristics (17). According to present data, L-FABP antisense up regulated inducible nitric oxide synthase along with formyl peptide recetor 1 (FPR1, ID: AA425767); both are ontologically related to NO signal transduction, and thus to progression of breast cancer (18, 19, 20) .

The miss-regulation of gene transcription has long been identified as a leading factor of oncogenesis; though its mode of approach to influence multiple pathways is till not very apparent. Telomerase reverse transcriptase (TERT), member of transcription regulating gene family, was down regulated in the presence of L-FABP antisense. Clinical studies identified TERT as telomerase-activator, a hallmark of tumorogenic progression especially in epithelial cells (21, 22). Another transcription regulating protein, zinc finger protein 212, was also down regulated. Recent evidence has recorded a higher growth rate of MCF-7/znf cells than that of the parental or the control cell lines (23). A present report suggests that blockage of L-FABP curbs histone deacetylase 1(HDAC1; ID: AA465353), a major component of Rb/E2f pathway, which functionally activates transcription and thus initiates cell cycle progression (24). Substantiating our previous study on the effect of L-FABP antisense on prostate cancer, a number of growth regulating genes appeared under-expressed in the present study too. Among them, transforming growth factor beta-2 (TGFB2, ID: AA233738) has been reported to posses an autocrine function in enhancing tumor growth and/or reducing immuno-surveillance of tumor development (25). Pathological studies accounted an over expressed insulin-like growth factor 1 (IGFR1,

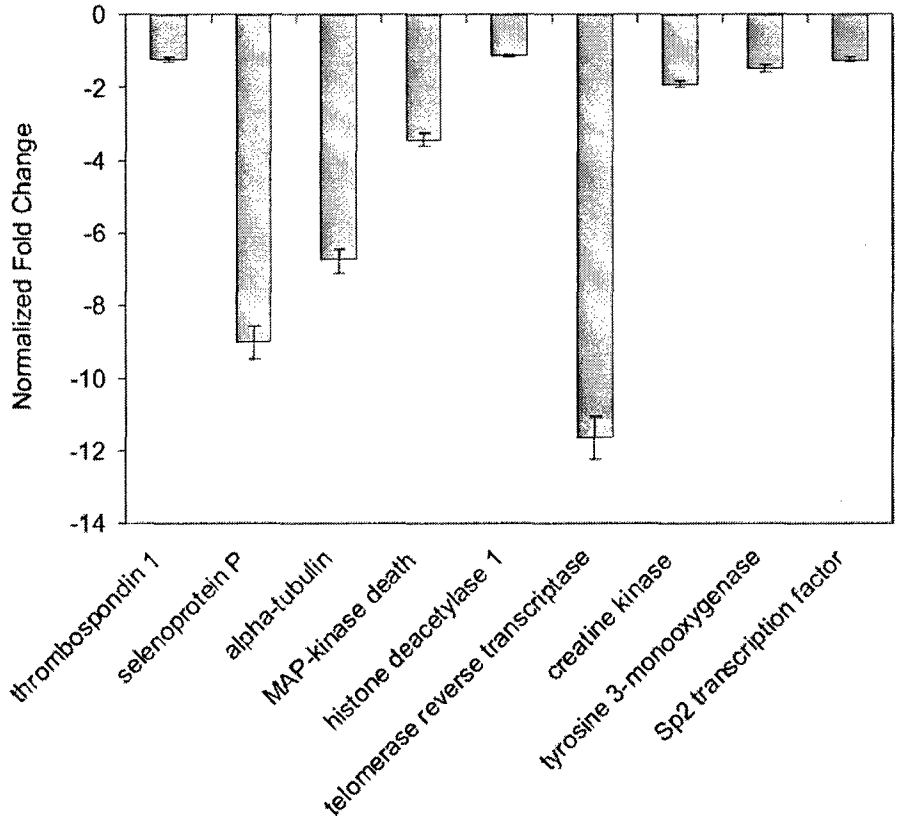

Figure 7. Confirmation of the expression patterns of genes shown to be down regulated by the antisense using cDNA microarray. Gene expression patterns were confirmed using real time PCR. Data shown are the expression levels after normalizing to actin and S18 genes.

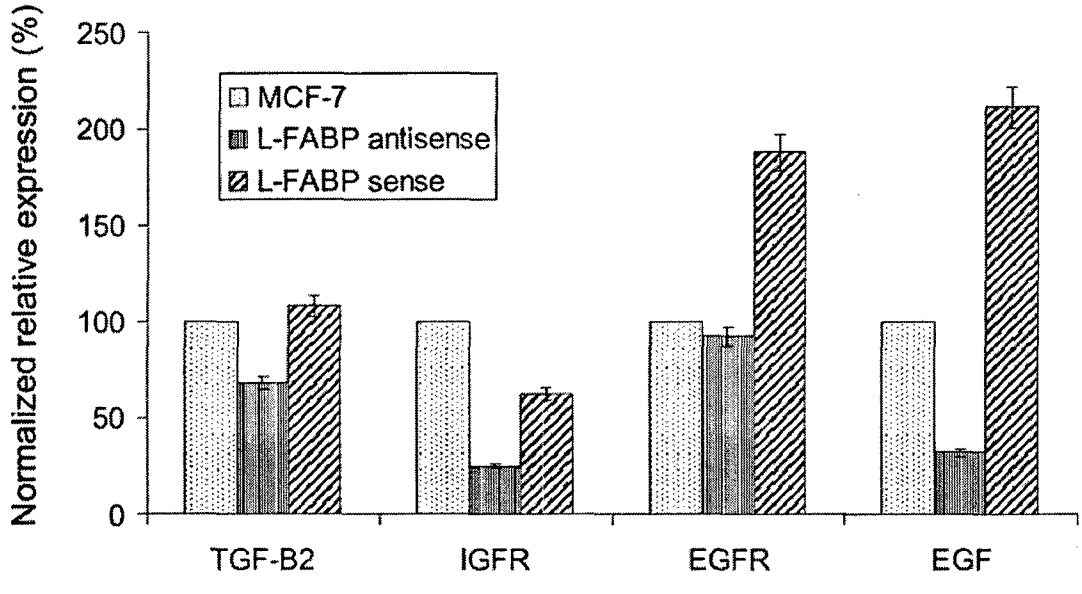

Figure 8. Confirmation of the expression patterns of genes shown to be up regulated by the antisense using cDNA microarray. Gene expression patterns were confirmed using real time PCR. Data shown are the expression levels after normalizing to actin and S18 genes.

ID: AA256532) in MCF-7 cell line where it executes anti-apoptosis effects by enhancing cell survival rate (25, 26, 28). Epidermal growth factor receptor (EGFR, ID: W48713), on the other hand, is not only involved in tumor cell growth, survival signaling, cell migration, metastasis formation and angiogenesis, but also seems to confer reduced responses of tumor cells towards anti-hormones (28, 29).

Interestingly, a few actins binding genes have emerged as significantly varying under the given treatment. Among them, Profilin 1 that has correlated to reduced growth rates and thus recognized as less tumorogenic (30), was up regulated. Beta-spectrin (SPTBN1, ID: W95287) and smoothelin (SMTN, ID: AA449234) take integral part in organization of the actins cytoskeleton; were down-regulated by the antisense treatment.

Three more down regulated genes in the present genome-wide study namely cysteine-rich angiogenic inducer 61 (CYR61, ID:AA777187), Thrombospondin 1 (THBS1, ID: AA464532), and SH2 domain protein 2A (SH2D2A, ID: AI003610) are ontologically linked with angiogenesis a key factor of growth, invasion and metastasis of malignant tumors. CYR61 induces angiogenesis by promoting vascularization as well as neovascularization (31). Intriguingly, it shares a histological resemblance, though in low level, with TSP1: an extracellular matrix protein, over expressed in breast tumor (32) and is well known for its anti-angiogenesis activity (33). Taken together, antisense of L-FABP might become a potent factor in anti-angiogenic therapy.

Prior data acknowledged FABPs as an instrumental factor in transport, storage and possibly in metabolism of fatty acids, the main energy source of most mammalian cells. Our previous study argued that though various FABPs share some functional and histological similarity, their effects on tumor cells are very different. L-FABP and I-FABP have been identified as pro-oncogenic in prostate cancer cells, while A-FABP and E-FABP showed anti-tumorogenic traits. In the present study, we proved that the aforementioned inference also holds true in mammary cells. Antisense treatment of L-FABP induces apoptosis in MCF-7, an epithelial breast cancer cell. Further investigation has revealed that L-FABP could possibly influence a number of pro-apoptotic genes and induce both intrinsic and extrinsic death receptor pathways. Moreover, it apparently plays instrumental role in cell proliferation, transcription and transduction pathways. Collectively, this study puts forward antisense of L-FABP as an important therapeutic agent to control tumor growth and to induce apoptosis. Together with our previous evidences regarding prostate cancer, L-FABP could be characterized as a potent target for effective cancer therapy.

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